CHARACTERIZATION OF THE MEMBRANE FRACTION ISOLATED BY THE FLUORESCEIN MERCURIC ACETATE TECHNIQUE OF BARLAND AND SCHROEDER

By

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A OISSERTATION PRESENTEO TO THE GRADUATE COUNCIL OF
THE UNIVERSITY OF FLORIDA
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE
DEGREE OF DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

197B

ACKNOWLEDGEMENTS

The author would like to thank Dr. Carl Feldherr in the Department of Anatomy as well as Mr. Jay Pomerantz for their assistance with transmission electron microscopy. For the scanning electron microscopy in this dissertation, the assistance of Dr. Werner Fischlschweiger in the Department of Basic Dental Science is greatly appreciated.

The author would especially like to express his gratitude to Dr. Kenneth D. Noonan for his encouragement throughout this project and his continuing enthusiasm for the author's medical as well as basic science training.

Part of the author's support during the last year of this research came from an institutional grant from the American Cancer Society which is gratefully acknowledged.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

BHK	baby hamster kidney
bis-acrylamide	N,N'-methylenebisacrylamide
CHO	Chinese hamster ovary
CMF-PBS	calcium, magnesium free phosphate buffered saline
CMF-PBS-EDTA-G	calcium, magnesium free phosphate buffered saline containing ethylenediamine tetraacetic acid and glucose
Con A	Concanavalin A
CPM's	counts per minute
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylenebis (oxyethelenenitrilo) tetraacetic acid
FMA	fluorescein mercuric acetate
g	gravity
IMP	intramembranous particle
LDL	low density lipoprotein
LETS	large, external, transformation-sensitive
MAS	membranes associated with substratum
MW	molecular weight
PAGE	polyacrylamide disc gel electrophoresis
PBS	phosphate buffered saline
PMSF	phenylmethylsulfonylfluoride
RNA	ribonucleic acid
SAM	substrate adherent material
SDS	sodium dodecyl sulfate
SEM	scanning electron micrograph
sptr	spontaneous transformant
TEM	transmission electron micrograph
TRIS	tris (hydroxymethyl) aminomethane
UMF	upper membrane fraction
WPM	whole plasma membrane

Abstract of Dissertation Presented to the Graduate Council
of the University of Florida in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy

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June 1978

Chairman: Kenneth D. Noonan
Major Department: Biochemistry

We have determined the composition of the membrane fraction isolated from a spontaneous transformant of Balb/c 3T3 cells by the technique of Barland and Schroeder. Using scanning electron microscopy, we have demonstrated that this upper membrane fraction (UMF) represents a topologically distinct membrane removed from an area circumscribing the nucleus. Our data suggest that the efficiency of membrane release achieved using this preparative technique depends on the morphology of individual cells.

We have found that the UMF represents a plasma membrane fraction which is depleted in lipids, glycoproteins, and high molecular weight peptides. We have compared the UMF isolate to the whole plasma membrane (WPM) fraction isolated using the technique of Brunette and Till and have shown that peptides and glycopeptides present in the WPM, but not found in the UMF, are retained by that part of the cell membrane which remains associated with the substratum following isolation of the UMF.

Our data clearly demonstrate that the UMF does not represent an isolate enriched in surface coat material. The data we have accumulated suggest that the compositional differences noted between the two membrane isolates (UMF and WPM) do not result from differential extraction of membrane components during the $ZnCl_2$-fluorescein mercuric acetate treatments required in the UMF isolation technique.

The data from these experiments strongly suggest that the UMF represents an area of plasma membrane which is compositionally and topologically distinct from the whole plasma membrane.

INTRODUCTION

It was known as early as the end of the 19th century that substances which were lipid soluble could penetrate the plasma membrane more easily than non-lipid soluble materials. For that reason, Overton[1] proposed in 1895 that the plasma membrane was composed of lipid layers. In 1925, Gorter and Grendel[2] suggested that the lipid portion of the erythrocyte membrane was composed of a double layer (bilayer) of lipid. Specifically, Gorter and Grendel demonstrated that a lipid monolayer prepared from acetone-extracted erythrocytes had an area equal to twice the surface area of the erythrocytes, hence suggesting that there was a double lipid layer around the cells. Their results, even though based on experiments now considered to have been technically questionable,[2] were a remarkable presage of events to come in the next forty years.

In 1935, Danielli and Davson suggested[3] a model of plasma membrane structure (the so-called "pauci-molecular model") which proposed that plasma membrane was composed of a central neutral lipid layer lined inside and out with an unknown number of layers of phospholipid plus adsorbed protein. Subsequently, Robertson[4] was convinced by electron microscopic evidence that the plasma membrane was composed of two lipid layers with closely adherent electron-dense material on both sides of the bilayer. Robertson's measurements suggested that the lipid bilayer itself was $\sim$40 Å thick and the adherent material 20 Å thick on each side of the lipid bilayer.

According to Robertson's original model, the inner layer of adherent material was composed of a monolayer of protein. On the basis of the data available to him, Robertson had to be less certain about the components of the outer layer, eventually describing them as "non-lipid."

In early work relating to membrane strucutre, it had been shown that the plasma membrane of the giant squid axon had a resistance of $\sim 10^3$ ohms/cm^2.[5] In 1963, Mueller and Rudin[6] demonstrated that the resistance of reconstituted bimolecular lipid membranes (extracted from bovine white matter) could be reduced from 10^8 ohms/cm^2 to about 10^3 ohms/cm^2 by the addition of proteins to the reconstituted membrane. The experiments of Mueller and Rudin[6] thus supported the previous models which described the plasma membrane as a lipid bilayer with adsorbed protein.

Finally, in the late 1960's a number of models were proposed which attempted to incorporate the growing data about the characteristics of cellular membranes into a meaningful framework which could account for the physiology of the organelles under consideration. Green and Perdue[7] suggested, on the basis of electron microscopic studies, that the mitochondrial membrane was composed of lipoprotein subunits fit together into a continuum. According to this model, all of the lipid and protein in the membrane was to be found in the repeating lipoprotein subunits, each of which contained a "basepiece" (an unvarying, membrane forming section) plus a "detachable sector" which was described as a projection from the basepiece. This "detachable sector" was considered to be an intrinsic part of the membrane but not essential to the continuity of the membrane.

According to Green and Perdue's model,[7] the repeating units of any given membrane were thought to be complementary in form (so that they would fit together to create a stable membrane) but different in composition and function.

Lenard and Singer[8] in 1966 developed a model which again considered the lipid bilayer to be the basic membrane structure to which other membrane components were "attached." Lenard and Singer suggested that the charged portions of the protein (as well as the lipid) were exposed on the exterior surfaces of the membrane. Their theory differed radically and importantly from previous models in the assertion that the nonpolar regions of the membrane peptides were sequestered in the interior of the membrane along with the nonpolar hydrocarbon tails of the phospholipids and other nonpolar lipids. Lenard and Singer[8] further suggested that structural membrane proteins would have specific amino acid sequences which would interact with both hydrophobic and hydrophilic areas of the membrane and that interactions of these types would in turn determine the conformational arrangement of the proteins.

The plasma membrane model most widely accepted at the present time was first proposed in detail by Singer and Nicolson in 1972[9] and is known as the fluid-mosaic model. The fluid-mosaic model amplifies the earlier proposal of Lenard and Singer.[8] Specifically, Singer and Nicolson envisioned a lipid bilayer in which amphipathic globular proteins were inserted to various depths (perhaps extending completely across the bilayer). Hydrophilic stretches of the membrane protein would, according to this model, be restricted to the inner or outer surface of the membrane, while hydrophobic areas of the proteins would

be sequestered within the interior of the lipid bilayer. Such an arrangement of lipids and globular proteins would, it was suggested, produce a thermodynamically stable continuum, something which the earlier models failed to do. A crucially important aspect of the Singer-Nicolson model was the suggestion that membrane proteins and lipids were free to move in the lateral plane of the membrane, subject only to protein-protein (and possibly protein-lipid) interactions which could act over short distances to produce physiologically important aggregates of membrane components. Furthermore, this model proposed that long range order (i.e., non-random arrangements of membrane proteins larger than oligomeric aggregates) would not be a property of the membrane itself but could occur only with the aid of components external to the membrane.[9]

The fluid-mosaic model suggested that there were two readily identifiable classes of membrane proteins. One class would include those proteins which were intimately associated, through hydrophobic and hydrophilic interactions, with the neutral and charged portions of the lipid bilayer. Such proteins were classified as intrinsic or integral proteins and were thought to be globular proteins, inserted into the lipid milieu of the membrane. Intrinsic proteins would require extensive disruption of the membrane continuum if they were to be removed from the plasmic membrane. The other class of membrane proteins tentatively identified by the fluid-mosaic model consisted of proteins which were more loosely attached to the membrane through noncovalent (electrostatic) interactions. These proteins were designated extrinsic or peripheral membrane proteins. Operationally, peripheral proteins were defined as proteins which could be separated from the membrane without disruption of the fluid bilayer.

Mobility of Plasma Membrane Constituents

Data derived in the 1970's concerning membrane structure and function have, for the most part, supported the basic tenets of the fluid-mosaic model. Modifications and extensions of the model deal primarily with constraints placed on the mobility of plasma membrane constituents in the lateral plane of the membrane. Several types of restrictions to lateral mobility have now become well recognized. As Singer and Nicolson suggested[9] and as Nicolson later demonstrated,[10] protein-protein interactions as well as protein-lipid interactions produce short range, ordered distributions of surface components (see also 11-21). Furthermore, loosely associated membrane proteins (extrinsic proteins)[22-35] and specific non-membranous components (e.g. the cytoskeleton)[36-60] also have been identified as possible mediators of the topological distribution of membrane peptides and glycopeptides. Our current concept of membrane protein arrangements suggests that the membrane is neither totally fluid nor totally fixed but exists in an intermediate state with some membrane constituents being fixed and some being mobile.

Protein-Protein Interactions

Noncovalent protein-protein interactions can produce planar aggregates of specific membrane components. Cell junctions are excellent examples of such physiologically significant oligomeric structures. Pappas[11] describes three major types of cell junctions while other authors[10,12] list four types:

1) Desmosomes apparently function mechanically to maintain cell-cell apposition. This class of junctions is formed by peripheral membrane proteins linking two separate membrane bilayers.

2) The tight junction, or zonula occludens, forms an impermeable barrier often completely surrounding the cell. Electron micrographs[11,12] suggest that the apposing membranes in the area of the tight junctions are firmly attached, leaving apparently no space between the associating cells.

3) The gap junction is a more complex specialization of membrane constituents which, as a result of the work of Goodenough and his collaborators,[13-15] is better characterized than the other junctions. The gap junction not only joins two plasma membranes, but also connects the cytoplasm of two cells. A gap junction consists of hexagonal arrangements of channels which are believed to be composed of integral membrane proteins. These channels allow only limited passage of materials larger than $\sim$15 Å between cells and apparently are responsible for electrolyte as well as non-electrolyte transport between cells.

4) The septate junctions[10,12] appear to be regionalized areas of membrane contact in which septa span the intercellular space. The function of this class of junctional complexes is not entirely clear but Staehelin[12] suggests that septate junctions may be involved in cell adhesion as well as cell-cell communication within cell layers.

Cell junctions are by no means the only examples of planar protein aggregates found in the plasma membrane. Another type of localized specialization, first described by Fawcett[16] is known as the coated region or coated pit.[17] This is a discrete area of membrane covered by electron-dense material. Although known to serve other purposes,[16] areas of the plasma membrane analogous to the coated pits have been identified by Brown and coworkers[17] as low density lipoprotein (LDL) receptors which also serve as sites for the rapid endocytosis of cholesterol in response to LDL binding.

Recently[18,19] it has been suggested that the formation and stability of protein-protein aggregates found on the surface of the plasma membrane might be mediated by disulfide crosslinks between membrane proteins. Such a mechanism for controlling membrane protein aggregation might be of particular significance to our work since a sulfhydryl reagent is employed as a "fixative" during our isolation procedure. This point will be developed further in the discussion below.

Protein-Lipid Interactions

Protein-lipid interactions are primarily understood in terms of hydrophobic and hydrophilic effects which produce a compartmentalization of individual membrane proteins into specific domains on the plasma membrane. Israelachvilli and coworkers[20,21] have recently devised a "unifying" membrane theory which proposes that interactions of nonpolar amino acids of intrinsic membrane proteins with the hydrophobic lipid milieu cause specific aggregations of proteins, both in the plane of the membrane as well as between proteins in opposite halves of the bilayer. These workers suggest that physical perturbations of the lipid molecules created by the presence of proteins in the lipid domain cause specific protein aggregations which in turn minimize the physical disturbance of the fatty acyl chain of the lipids. This hypothesis, although unproven experimentally, is certainly consistent with what is known about membrane thermodynamics.

Peripheral Proteins and Restraints on Mobility

As Singer and Nicolson[9] suggested, peripheral (or extrinsic) proteins may play a role in controlling the topological distribution of surface proteins. The premier example of a peripheral protein

controlling mobility of integral membrane proteins may be found in the work relating to spectrin, which is an extrinsic membrane protein localized on the inside of the erythrocyte membrane.[10,22-27] Spectrin is believed to be responsible for such diverse properties of the erythrocyte as maintenance of the biconcave shape of the cell as well as the restriction of surface complex mobility.[10,22,23] Elgsaeter and Branton[24] have demonstrated that removal of spectrin from purified erythrocyte "ghosts" produces a loss of the normal disk-like shape. More interestingly with regard to controlling membrane peptide mobility, Nicolson and Painter[25] have shown that antibodies prepared against spectrin (which can aggregate spectrin molecules on the inside of the erythrocyte ghost) produce a marked aggregation of glycoproteins localized to the exterior of the intact erythrocyte.

Other laboratories, including those of Yamada and Weston[28] and Hynes and Humphreys,[29] have studied an external, peripheral protein which may play a role in determining the topological distribution of fibroblast membrane proteins. This protein, known as the large, external, transformation sensitive (LETS) protein[29] or as cell surface protein,[28] is a glycoprotein of $\sim$250,000 molecular weight which has been localized to the outer cell surface. Using antibodies directed against LETS, Mautner and Hynes,[30,31] as well as Chen and coworkers,[32,33] have localized the protein to the peripheral and lower cell surfaces and have shown it to be present in areas of cell-cell contact. Albrecht-Buehler and Chen[34] have proposed a role for the LETS protein in determining the mobility of large particles attached to the cell surface while Schlessinger et al.,[35] using photobleaching techniques, have demonstrated that the LETS protein itself is immobile and that

crosslinking of another surface protein or glycoprotein to LETS immobilizes the cross-linked surface component.

Extramembranous Restraints on Mobility

The initial formulation of the fluid-mosaic model[9] suggested that long range order of membrane proteins would be maintained primarily through interactions of the membrane constituents with cellular components which are not themselves components of the plasma membrane. The cytoplasmic elements which have been implicated in control of membrane peptide or glycopeptide components are part of the cell's cytoskeleton and include microfilaments, thick filaments, and microtubules. Wolosewich and Porter,[36] employing the technique of high voltage electron microscopy, have recently demonstrated that in the cytoplasm the cytoskeletal elements are arranged as a lattice which courses throughout the cytoplasm and appears to interact with the plasma membrane as well as other cytoplasmic organelles.

Recently it has become clear that the microfilaments are actin-like polymers[10,37-41] and that thick filaments may be composed of myosin-like polymers.[10,38,40-43] These filaments are arranged in structures of variable dimensions which are often found closely apposed to the plasma membrane.[44,45] Microtubules, on the other hand, have been demonstrated to be assemblies of tubulin,[10,46,47] a protein of molecular weight 100,000 which spontaneously aggregates at a distinct pH, temperature and calcium ion concentration.[43,47,48]

Although the purported interaction between cytoskeletal elements and membrane components is far from being understood, a working hypothesis[10] has evolved which suggests that microfilaments and thick filaments have a contractile role and microtubules a purely skeletal

role. This model suggests that microtubules and microfilaments interact with each other and together form attachments with proteins of the inner membrane surface. The components to which the cytoskeletal elements are attached may be integral proteins or peripheral proteins (such as α-actinin)[49] which in turn may act to immobilize a number of integral proteins.

It has been postulated that, as a result of interactions between the cytoskeleton and membrane components, certain membrane components are maintained in a random distribution.[50,51] Other evidence has clearly demonstrated an important role for the cytoskeleton in maintaining the random distribution among surface constituents.[52,53] Specifically it has been suggested that these cytoskeletal elements may act to limit the capacity of protein-protein interactions for clustering surface proteins. This aspect of cytoskeletal control over surface proteins has been investigated with regard to intramembranous particle (IMP) clustering and Concanavalin A (Con A) receptor distribution using agents which disrupt cytoskeletal elements.[52-54] Intramembranous particle distributions have been studied using freeze fracture replicas of mouse embryo fibroblast (3T3) cells[52] and Con A receptor movements have been studied in these and other cells using fluorescent and ferritin labeled lectins.[53-55] In this work it has been demonstrated that both intramembranous particles and lectin receptors are distributed randomly on the surface of 3T3 cells.[52-55] Poste et al.[52] have demonstrated that this random distribution of surface glycoproteins and intramembranous particles is dependent on the maintenance of an intact cytoskeletal array. From the data of Poste and his collaborators,[52] as well as many other investigators,[10,53-55] it is clear that

disruption of the cytoskeletal elements permits the lectin receptors to be moved from a random array into a clustered distribution.

A close physical association of cytoskeletal elements and the plasma membrane has been demonstrated, especially in the areas of cell-cell and cell-substratum contact.[44,45] Specifically, Revel et al.[56] have suggested that the "sole plate," as the area of cell-substratum adhesion is called, is reinforced by the arrangement of intracellular cytoskeletal elements in the area. Revel and Wolken[57] have shown, using baby hamster kidney (BHK) cells, that many microfilament bundles are detectable in the area of the "sole plate" and that the bundles are oriented along the long axes of the cell. Furthermore, Harris[58] and others[59,60] have suggested that membrane movements such as ruffling[59,60] and particle transport[58] may be mediated through the interactions of contractile cytoskeletal elements with the plasma membrane.

Surface Specializations in Cultured Cells

The constraints on the lateral mobility of membrane proteins which have been discussed above act *in vivo* and *in vitro* to produce specific arrangements of surface proteins. These arrangements may vary even within the same cell, if different parts of the cell face different environments. An example of such an occurrence *in vivo* is the rat hepatocyte, in which investigators have shown[61,62] that the sinusoidal surface membrane differs in composition from the plasma membrane on the canalicular side. Similarly, cultured cells grown attached to a substratum clearly come into contact with at least three different "environments" -- the solid substratum, the fluid medium, and contacts with other cells. Due to the different milieus contacted by the

different areas of the cells, one might anticipate that differences in the membrane composition exist among these different areas. The lower cell surface, as already mentioned, displays the so-called "sole plate," which is apparently a membrane specialization involved in cell-substratum adhesion.[56,57] Cell-cell interactions occurring at the cell periphery involve such specializations as the already described gap junctions, desmosomes, tight junctions and septate junctions.[10-15] Finally, the upper cell surface, which has been little investigated until now, interacts with a fluid medium and might be expected to be differentiated so as to perform functions not required of the lower or peripheral portions of the cell. Our interest has been to more clearly define the differences, if any, in the biochemical composition of the upper and lower surface membranes in cultured cells grown attached to a solid substratum.

Our interest in the difference between the biochemical structures of the upper and lower cell surfaces has been stimulated by other studies which have clearly demonstrated functional differences between these areas of the cell membrane. Specifically, a number of laboratories[56,57,63-75] have investigated the physiology of cell-substratum adhesion, a function of the lower cell surface. Other investigators[56,57,76-83] have investigated morphological differences observed between the upper and lower cell surfaces of cells attached to a substratum.

Cell-Substratum Adhesion

Many cell types can attach very firmly to solid substrata. The study of the plasma membrane's role in this attachment process has improved our understanding of cell surface specializations. Rajaraman

et al.[63] have studied the phenomenon of attachment using scanning electron microscopy. They have demonstrated four distinct phases in the process of cell-substratum adhesion:

1) Relatively spherical cells make initial contact with the substratum.

2) Filopodia then shoot out of the contacting cells in all directions to establish adhesions around a broad periphery.

3) Cytoplasm spreads out into the filipodia, eventually covering the area of adhesion.

4) Finally, the central area of the cell flattens out.

Grinnell and coworkers,[64-68] Heckman et al.,[69] Nath and Srere,[70] and Juliano[71] have all initiated studies into the molecular mechanisms underlying cell-substratum contact and adhesion. Grinnell has suggested,[64] as a result of studies in baby hamster kidney (BHK) cells, that binding to the substratum, cell attachment, and spreading on that substratum are distinct phenomena. Grinnell has also demonstrated[64] that components of the tissue culture medium (such as calf serum) radically affect certain steps in the attachment adhesion processes. Specifically Grinnell has demonstrated that although cell binding to the substratum occurs in the absence of calf serum, adhesion and spreading occur only in the presence of calf serum.

Further data relating to the role of serum components in adhesion have been supplied by Heckman et al.[69] and Revel and Wolken.[57] These investigators have demonstrated that there is a thin, electron dense layer of material, localized between the cells and substratum, whenever serum is a component of the medium. This layer is found on the substratum whether or not cells are present and therefore is believed to

represent a serum precipitate to which cells attach and upon which cells spread.

A number of workers have studied the surface specializations thought to be involved in cell-substratum adhesion. In experiments in which cells were washed off the substratum with streams of medium, Revel et al.[56] demonstrated that, following removal of the cells from the substratum, small patches of membranous material remain attached to the substratum. These attachments are believed to have been localized to various adhesive areas on the underside of the cell as well as to the lamellipodia.[56] Harris[72] and Revel et al.[56] have both shown that the retraction fibers observed when cells round up (e.g. in mitosis or after trypsinization) are associated with the attachment points observed in flattened cells. All of these adhesive points probably represent the material which Culp[73,74] calls substratum-attached material (SAM), which has been extensively investigated by Culp and co-workers (see below).

Juliano's investigations[71] in Chinese hamster ovary (CHO) cells have shown that the carbohydrate moieties of surface molecules are not involved in the initial phases of cell-substratum adhesion, but may act to modulate the susceptibility of the attachment processes to proteases. Curtis et al.[75-77] have reported that alterations in plasma membrane lipids cause well-defined changes in cell adhesion, and that these alterations in lipid composition produce changes in bulk membrane properties. These results suggest that changes in membrane fluidity affect cell adhesion. The work of Grinnell and coworkers[64-68] as well as Juliano[71] had further shown that the serum-dependent attachment of cells is trypsin-sensitive, strongly supporting their assertion that proteins have a central role in cell-substratum adhesion.

Upper and Lower Surfaces - Structural and Functional Differences in Cultured Cells

The extensive work directed at elucidating the molecular mechanisms underlying cell-substratum adhesion has led to numerous examples of differences between the upper and lower surfaces of cells cultured on solid supports. Revel and coworkers,[56,57] in electron microscopic studies of the underside of cells as well as the points of cell-substratum contact have been able to show striking differences between the two opposing surfaces. Differences observed include the presence of "sole plates" on the lower cell surface as well as differences in the general appearance of the two cell surface compartments, including the lack of microvilli on the lower surface of L cells. Similar findings have been reported by Brunk et al.[78] who have investigated the adhesion of a human glial-like cell line to the substratum.

Culp[73,74] and coworkers have studied the properties of substrate adherent material (SAM), which is a layer of firmly bound protein, glycoprotein, and glycosaminoglycan left behind on glass or plastic substrata after mouse fibroblasts are removed from the substratum by repeated washes with ethylenebis (oxyethelenenitrilo) tetraacetic acid (EGTA). SAM has been shown to be composed of small peptides linked to long chains of hyaluronic acid[73] and is unequivocally identifiable as a product of cellular metabolism rather than a serum precipitate.[74] SAM has also been localized to "focal pools" found only on the lower surface of the cell.[74] The best available evidence suggests that these "focal pools" represent the sites of attachment of the retraction fibers identified by Revel and his collaborators[56,57] (see above).

Abercrombie and colleagues,[59,79,80] Harris,[58,72] and Ingram[81] have all investigated the movement of particles on the upper surface of a moving cell. Their studies have shown that particles which are attached at the periphery of a cell are moved in toward the center of the cell (centripetal flow) from any point of attachment. Although Harris and Dunn[82] have demonstrated that there may be some particle flow on the ventral surface of cells, all these studies[58,59,72,79-82] show a marked preference for a dorsal movement of attached particles, suggesting that the upper surface of the cell possesses a function (and therefore probably a structure) unique to that particular compartment of the cell membrane.

DiPasquale and Bell[83] have shown that in many different cell lines, cells will not spread onto or move over the upper surface of their neighboring cells. These authors have suggested that the inability of the upper cell surface to support spreading could be a generalized phenomenon related to the molecular composition of the upper surface of the cell. In addition, DiPasquale and Bell[83] have demonstrated that particles can be attached to the cell surface only at the cell periphery and assert that the failure of particles to adhere directly to the upper surface of the cell is suggestive of variations in the relative adhesiveness of different points along the cell surface.

Isolation of Specific Plasma Membrane Regions

In order to biochemically characterize the different areas of the cell surface, it is necessary to separate specific plasma membrane regions from the cell and from the remainder of the cell's plasma membrane. A number of techniques have been developed for the isolation

of some "specific" plasma membrane fractions. Cohen et al.[84] have isolated plasma membranes from HeLa cells after first binding the cells to polylysine-coated polyacrylamide beads and then lysing the cells while still attached to the beads. This procedure leaves at least part of the plasma membrane associated with the polylysine coated bead. The membrane stuck to the bead can subsequently be isolated by centrifugal density separation. Jacobson[85] further developed this technique in an attempt to optimize purity and yield of the isolate. Lutz, Liu, and Palek[86] have isolated spectrin-free vesicles which are spontaneously released from human erythrocytes during incubation of the red cells in glucose-free medium. Scott[87] has demonstrated that vesicles are released from a variety of cell types by treatment with formaldehyde, other related aldehydes, and disulfide blocking agents. Vandenburgh[88] has studied the plasma membrane "blisters" which are released from myoblasts and fibroblasts after treatment of these cell types with glycerol. Van Blitterswijk et al.[89] have studied membranes exfoliated from normal and leukemic lymphocytes and discovered that the spontaneously released membranes differ from plasma membrane isolated from the cells themselves. Similarly, DeBroe et al.[90] have studied spontaneously released plasma membrane fragments isolated from human body fluids as well as from HeLa cells in culture.

Although these techniques allow the isolation of specific regions or fragments of plasma membrane, they all suffer from the drawback that the location of the membrane on the cell prior to release is unknown. Furthermore, in those isolation procedures employing blistering or vesiculation it is unclear whether cytoplasmic components

are trapped in the vesicles prior to release of the vesicles from the cell.

We have been interested in determining the biochemical composition of the upper and lower plasma membranes in substratum-attached cells. Our investigation began with the study of a technique which had previously been reported[91] to strip surface membranes from cells attached to substratum without disruption of the adhesion between the lower surface and the substratum. This technique, first introduced by Barland and Schroeder[91] as a method for the rapid isolation of membranes from cultured cells, had not been rigorously investigated prior to our studies to determine whether or not a specific region of the cell surface was removed during the isolation technique. In addition, no one had attempted to compare the surface membrane isolated by this technique to membranes isolated using other procedures. Although we recognized that this technique had some drawbacks, including the need for pretreatment of the cells with chemicals which might alter the topologic distribution of membrane components, we felt that the technique warranted further investigation because it might offer us the opportunity to study a membrane fragment removed from a well-defined area of the cell which had not been intensely investigated with regard to its composition.

MATERIALS AND METHODS

Materials

Tissue culture media and sera were purchased from Grand Island Biological Company (Grand Island, N.Y.). Tissue culture flasks and dishes were obtained from Corning Glass Works (Corning, N.Y.). Reagents used for electrophoretic analysis of plasma membrane and cellular peptides and glycopeptides were products of Bio-Rad Laboratories (Rockville Center, N.Y.). Scintillation fluid and all radioisotopes except ^{125}I were purchased from Amersham/Searle Corp. (Arlington Heights, Ill.). ^{125}I was obtained from Schwarz/Mann division of Becton, Dickinson and Co. (Orangeburg, N.Y.). Lactoperoxidase was a product of Calbiochem (La Jolla, Calif.). Concanavalan A was obtained from Miles-Yeda (Miles Laboratories, Inc., Elkhart, Ind.) and then affinity purified on Sephadex G-100. Dextran T-500 and Sephadex G-100 were from Pharmacia (Piscataway, N.J.). Cytochrome c and phosphorylase A, which were used as molecular weight standards, as well as fluorescein mercuric acetate (FMA), phenylmethyl sulfonyl fluoride (PMSF), and sodium metaperiodate were obtained from Sigma Chemical Co. (St. Louis, Mo.). Reagents for electron microscopy were purchased from Polysciences, Inc. (Warrington, Pa.). Collagenase, as well as most of the proteins used as molecular weight markers in electrophoresis, were purchased from Worthington Biochemicals (Freehold, N.J.). All other laboratory reagents were purchased from Scientific Products (Ocala, Florida).

Methods

Maintenance of the Cell Line

The cell line used in this work was a spontaneous transformant of a Balb/c 3T3 mouse embryo fibroblast cell line (Clone A-31). This transformant was isolated in our laboratory and will be referred to as sptr 3T3 throughout this dissertation. The cells were maintained in 75 cm^2 tissue culture flasks in Oulbecco's modified Eagle's medium supplemented with 10% (v/v) calf serum plus 1% (v/v) penicillin-streptomycin (final concentrations 1000 units/ml penicillin, 100µg/ml streptomycin). Cells were passaged every 3 days at approximately 80% confluency. The cells were routinely tested for pleuropneumonia-like organism contamination by both autoradiography[92] and nutrient agar.[93] Cells were discarded after approximately 30 passages and fresh cell lines started from stocks frozen at an early passage number. Under the growth conditions used, the sptr 3T3 cells grew to a final density of approximately 1×10^5 cells/cm^2. In all of our experiments, cells were used when they reached a density of approximately 8×10^4 cells/cm^2. Cells to be used in the experiments described were routinely grown on 75 cm^2 plastic tissue culture flasks; however, in some experiments cells were grown in 78.5 cm^2 tissue culture dishes or 180 cm^2 glass Blake bottles.

Membrane Isolation

A. Upper membrane fraction (UMF). sptr 3T3 cells were grown to 80% confluency on plastic tissue culture flasks or glass Blake bottles. The medium was decanted and the cells washed three times with either phosphate buffered saline (PBS, pH 7.2) or 1.6×10^{-1} M NaCl plus 9×10^{-3} M $CaCl_2$ at 37°C. Following the initial washes the cells

(still attached to the substratum) were treated for 10 minutes at room temperature with 1 x 10^{-3} M $ZnCl_2$ in dimethyl sulfoxide (DMSO; volume ratios 4:1). Ten milliliters of $ZnCl_2$-DMSO were used when membranes were to be isolated from cells attached to 75 cm^2 flasks while 30 ml of $ZnCl_2$-DMSO were used if cells were grown in Blake bottles. Following this "tanning" process, the $ZnCl_2$-DMSO was removed and ice-cold FMA ($\sim$2.2 x 10^{-3} M) in 2 x 10^{-2} M Tris-HCl (pH 8.1) was added to the cells (20 ml for flasks, 60 ml for Blake bottles).

> The fluorescein mercuric acetate (FMA) solution was prepared by a-ding 1.87g of FMA to 1L of 2 x 10^{-2} M Tris-HCl, pH 8.1. The FMA solution (2.2 x 10^{-3} M) was stirred overnight at room temperature, filtered through Whatman #1 paper, then incubated at 37°C for 24 hours. The resulting solution was refiltered and the FMA stock was stored in the cold.

The flasks or bottles were placed on ice and shaken for 40 minutes on a rotatory shaker at 120 RPM. The shearing force of the solution removed a membranous fraction from the cells which could be decanted along with the FMA. (Prolonged shaking of the cells in FMA released intact cells into the FMA while shorter exposure to the shearing action reduced the yield of membrane.) After decanting the FMA solution the membrane sheets were pelleted at 600 x g for 10 minutes in an HB-4 rotor (in the cold). This pellet was washed twice with cold 1 x 10^{-3} M $NaHCO_3$, resuspended in 5 ml of 25% (w/v) sucrose, layered onto 20 ml of 50% (w/v) sucrose, and finally centirufged for 1 hour at 370 x g (also in the cold). The membrane sheets spun through both concentrations of sucrose and were collected in a pellet. The membrane pellet was then washed in 1 x 10^{-3} M $NaHCO_3$ and the purified UMF collected at 7000 x g for 10 minutes.

Attempts to isolate UMF from sptr 3T3 cells without the $ZnCl_2$-DMSO treatment or by using $ZnCl_2$ without DMSO were unsuccessful. Similarly the omission of FMA prior to shaking the cultures failed to produce membrane sheets. Also, attempts to isolate UMF from cells grown on 78.5 cm^2 tissue culture dishes were unsuccessful in that the yield of membrane was very low.

B. Membranes associated with substratum (MAS). The material remaining adherent to the substratum after the removal of UMF was washed five times with ice-cold PBS and then scraped off into PBS with a rubber policeman. This material was washed three more times with ice-cold PBS, then incubated at 37°C for 15 minutes in 1×10^{-2} M Tris-HCl containing 1×10^{-3} M EDTA (pH 7.5). The material was then homogenized in a tight fitting Dounce homogenizer to shear the membranes free of nuclei and other contaminating intracellular organelles. The resulting homogenate was centrifuged at 4000 x g for 10 minutes. The pellet was resuspended in 65% (w/w) sucrose made up in 1×10^{-2} M Tris-HCl containing 1×10^{-3} M EDTA and overlayered with a 60% (w/w), 55% (w/w), and 35% (w/w) sucrose step gradient. This gradient was spun for 3.5 hours at 100,000 x g in a Beckmann SW-41 swinging bucket rotor. The membranous material was collected from the 35%/55% and 55%/60% sucrose interfaces diluted with PBS and pelleted at 12,000 x g.

C. Whole plasma membrane (WPM). sptr 3T3 cells were removed from the tissue culture dish by washing the plates three times with calcium, magnesium free phosphate buffered saline (pH 7.2) containing 5.5×10^{-2} M ethylene diaminetetraacetic acid (EDTA) and 6.1×10^{-3} M glucose (CMF-PBS-EDTA-G) and then incubating the cells in CMF-PBS-EDTA-G for 15 minutes at 37°C. We found that the addition of glucose to CMF-PBS-EDTA

increased the rate at which the sptr 3T3 cells were released from the substratum and improved the relative viability of the cells released.

Following release from the substratum the cells were washed three times with PBS and then a sub-cellular fraction enriched in plasma membrane was isolated via the aqueous two-phase polymer system described by Brunette and Till.[94] Membranes were banded at least three times in the aqueous polymer and then washed with PBS for 10 minutes to remove excess polymer. In those experiments where enzyme activity was monitored, the final membrane fraction was washed with 1×10^{-3} M Tris, 1×10^{-2} M EDTA (pH 7.5) instead of PBS to remove excess zinc.[95]

Electron Microscopy

Samples were prepared for transmission electron microscopy (TEM) by fixation (2 hours, 3 changes) in 2% (v/v) glutaraldehyde made up in 1×10^{-1} M phosphate buffer (pH 7.4). The samples were then washed extensively in 1×10^{-1} M phosphate buffer (pH 7.4) and treated with 1% (v/v) osmium tetroxide in 1×10^{-1} M phosphate buffer (pH 7.4) for 15 minutes. Samples were then dehydrated through successively increasing concentrations of ethanol. Samples were embedded in Spurr's low viscosity medium[96] and sectioned. Silver sections were post-stained with uranyl acetate and examined with a Siemens Elmiskop 101 at 80KV.

For scanning electron microscopy (SEM) cells were grown on 10 x 60 mm glass coverslips until approximately 80% confluent. The cells were washed three times with PBS and then processed through the various steps described above for UMF isolation. Individual samples were fixed after each step in the procedure. The samples were fixed

overnight in 1×10^{-1} M cacodylate buffered (pH 7.4) glutaraldehyde [2.5% (v/v)] and then dehydrated through successively increasing concentrations of ethanol. Following dehydration the cells, still attached to coverslips, were critical point dried, mounted, and coated with gold and palladium in a "Hummer II" coating apparatus. The samples were examined in a Zeiss Novascan scanning electron microscope.

SDS Polyacrylamide Gel Electrophoresis

Peptides and glycopeptides were separated using the discontinuous SDS-polyacrylamide gel system (SDS-PAGE) designed by Laemmli.[97] The slab gels used throughout this work consisted of a running gel of 7.5-12.5% (w/v) acrylamide and a 5.6% (w/v) stacking gel. The acrylamide to bis-acrylamide ratio was 37.5:1 in both the stacking and running gels. The gradient gel efficiently separated membrane components having MW's between $\sim$300,000 and 15,000. After completion of the electrophoretic separation, the slab gels were immediately fixed in 10% (w/w) trichloroacetic acid, stained with coomasie brilliant blue, and destained according to the technique of Weber and Osborn.[98]

Autoradiography and Fluorography

[^{125}I]-labeled components separated by SDS-PAGE were identified using autoradiography. Gels were prepared for autoradiography by equilibration in 3% (v/v) glycerol in water for 30 minutes followed by drying. After drying, gels were placed into light-tight carriers with x-ray film (Kodak XRP-1) for exposure at room temperature.

The distribution of [^{3}H]-labeled components separated on SDS-PAGE slab gels was displayed via the fluorographic technique of Bonner and Laskey.[99]

Radioisotope Incorporation

A. Metabolic labeling. Proteins were metabolically labeled by maintaining cells for 72 hours in medium containing 2.5μCi/ml L-[4,5-^{3}H] leucine. Glycoproteins were metabolically labeled by maintaining cells in medium supplemented with 2.5μCi/ml D-[1-^{3}H] glucosamine hydrochloride or 1.0μCi/ml L-[6-^{3}H] fucose. Cells were maintained for 72 hours in 5.0μCi/ml L-[G-^{3}H] proline in an attempt to determine the contribution of collagen to the UMF. Phospholipids were labeled by maintaining the cells for 72 hours in medium containing 2.5μCi/ml [methyl-^{3}H] choline chloride, 0.1μCi/ml [U-^{14}C] glycerol, 2.5μCi/ml [1-^{3}H] ethan-1-ol-2-amine hydrochloride, 0.63μCi/ml myo-[2-^{3}H] inositol, or 2.5μCi/ml [9,10(n)-^{3}H] palmitic acid. Sulfate containing components were metabolically labeled by maintaining the cells for 72 hours in medium supplemented with 100μCi/ml [^{35}S].

B. Surface labeling. In all of our attempts at preferentially labeling surface components of the plasma membrane, cells were radiolabeled while still attached to the substratum although removal of the cells from the substratum with CMF-PBS-EDTA-G did not markedly influence the pattern of labeling. Before labeling the cells were washed three times with PBS. Proteins were labeled with 400μCi/ml [^{125}I] using lactoperoxidase and hydrogen peroxide as described by Phillips and Morrison.[100] Sialic acid residues were labeled using sodium metaperiodate/sodium boro [^{3}H] hydride by incubating the cells in calcium, magnesium free PBS (CMF-PBS, pH 7.4) containing 1 x 10^{-3} M sodium metaperiodate for 20 minutes at 0°C. The cells were washed twice with PBS and then incubated 20 minutes at room temperature in PBS plus 1.25mCi/ml sodium boro [^{3}H] hydride. The cells were then

washed two times more with PBS before initiating membrane isolation. Surface galactosyl residues were labeled with sodium boro [^{3}H] hydride following galactose oxidase oxidation using the technique described by Critchley.[101]

C. [^{125}I] Concanavalin A binding to glycopeptides. After SDS-PAGE, gels were fixed, stained, destained, and then Concanavalin A (Con A)-binding glycopeptides were labeled in the gel with affinity-purified [^{125}I] Con A as described by Burridge.[102] The Con A binding glycoproteins were displayed by preparing an autoradiograph of the dried gel.

D. Iodination of calf serum. Fifty milliliters of calf sera were incubated with 10μCi/ml [^{125}I] and labeled according to the lactoperoxidase catalyzed technique of Phillips and Morrison.[100] After iodination the serum was dialyzed for 48 hours at 4°C against 2L of PBS wich changes every 12 hours. After dialysis, the specific activity of the labeled serum was 4.3 x 10^{4} cpm's/mg protein (6.5 x 10^{6} cpm's/ml serum). The labeled serum supported cell growth as effectively as unlabeled serum.

Protein Determination

Protein concentration was determined using the procedure of Lowry et al.[103]

Phospholipid Determination

The phospholipid content of the UMF and WPM was determined from a chloroform-methanol[104] extract of the two membrane fractions. Lipid phosphorus was determined using an ascorbate-phosphomolybdate assay[105] and a factor of 25 was used to convert micrograms phosphorus to micrograms phospholipid.

RNA and DNA Determinations

Due to the interference of the brilliant orange FMA with both the diphenylamine[106] and acid orcinol[107] assays, it was necessary to combine these assays with [^{3}H] thymidine of [^{3}H] uridine labeling of the cells in order to determine the relative contamination of the various membrane fractions with DNA or RNA. To determine DNA contamination of the membrane fractions, cells were grown for 72 hours in the presence of 2.5μCi/ml [methyl-^{3}H] thymidine. Aliquots of the cells were lysed and the specific activity of the DNA determined via the diphenylamine[106] assay. The calculated specific activity was then used to determine the relative amount of DNA associated with the various membrane fractions. The relative contamination of the membrane fractions with RNA was determined in the same manner except cells were grown for 72 hours in [5,6-^{3}H] uridine and the specific activity determined using the acid orcinol[107] technique.

Vinblastine Sulfate, Cytochalasin B Treatment

In order to determine what role, if any, the cytoskeletal elements played in determining the composition of the UMF subfraction of the plasma membrane, sptr 3T3 cells grown to 8×10^4 cells/cm^2 were treated with 2.5×10^{-7} M vinblastine sulfate for 2 hours at 37°C or for 45 minutes at 37°C with 8×10^{-6} M cytochalasin B. Following this treatment the cells were washed three times in PBS then the UMF was isolated as described above.

Enzyme Assays

Na^+, K^+ ATPase was measured according to the technique described by Brunette and Till.[94] Glucose-6-phosphatase activity was measured according to the procedure of Franke et al.[108] Inorganic phosphate

release in both enzyme assays was measured via a modification of the Fiske-Subbarowe[109] technique.

RESULTS

As indicated in Figure 1A, and has been demonstrated by others,[91,110] pretreatment of sptr 3T3 cells with $ZnCl_2$/DMSO-FMA followed by agitation in a rotatory shaker releases membranous material from cells attached to the substratum. We have designated this material as the upper membrane fraction (UMF) since the majority of the cell remains associated with substratum following the isolation of UMF (see Figures 2B and 3F). Approximately 4% of the total cell protein is isolated as the upper membrane fraction.

The membranous material isolated by our modification of Barland and Schroeder's[91] original technique represents a membrane fraction free, as judged by electron microscopy (Figure 1A), of any obvious contamination with other cellular organelles. The only apparent contaminants of the UMF are particles adherent to one side of the membrane. These particles have the approximate dimensions of cytoplasmic ribosomes and might result from the adhesion of cytoplasmic material (see Figure 1B) to the inside of the plasma membrane. However, despite the apparent association of ribosome-like particles (or alternatively, other ground substance) with the UMF it is clear from the data in Table I that the contamination of the UMF with both DNA and RNA is very low.

After removal of the UMF, the material remaining attached to the substratum includes intracellular organelles (most notably nuclei), as well as portions of the plasma membrane (see Figures 3E and 3F), most

Table 1

Relative Purity of Membrane Isolates

Membrane Fraction	Na^+, K^+ ATPase µmoles Pi/mg protein/hour	Glucose-6-Phosphatase µmoles Pi/mg protein/hour	ONA µg/mg protein	RNA µg/mg protein
WPM	7.65	0.43	25.0	150.6
UMF	N.O.	N.D.	2.9	2.8
MAS	N.O.	N.O.	6.9	24.8
homogenate	0.51	0.59	117.3	175.3

Na^+, K^+ activated ATPase was assayed according to the technique outlined by Brunette and Till.[94] Inorganic phosphate release was measured according to the method outlined by Chen et al.[109] Glucose-6-phosphatase activity was determined according to the technique of Franke et al.[108] ONA was determined in [^{3}H] thymidine labeled whole cell homogenates via the technique of Burton.[106] The contamination of the UMF and MAS with DNA was determined from the CPM's [^{3}H] thymidine associated with the respective membrane fractions and then working back to the relative contamination using the pre-determined specific activity of ONA. RNA was determined in [^{3}H] uridine labeled whole cell homogenates by the acid-orcinol procedure.[107] RNA contamination of the membrane fractions was determined in the same way as the ONA contamination using the specific activity of RNA. N.O.: not determined because the cells had been treated with $ZnCl_2$/OMSO-FMA which destroys enzyme activity.

notably those areas of the plasma membrane localized to the underside of the cell and the cell periphery. A membranous fraction has been isolated from the material remaining associated with the substratum by scraping the residual substratum-adherent material into ice-cold PBS and then proceeding with the isolation as described in the Materials and Methods section. The membrane enriched material derived from this substratum-adherent component bands at the 35%/55% (w/w) sucrose and 55%/60% (w/w) sucrose interfaces from which it is collected and pelleted. This isolated fraction has been designated "membranes associated with substratum" (MAS). Figure 1C is a transmission electron micrograph of the MAS. This material is membranous but, unlike the UMF, the MAS has heterogeneous fibrous material adherent to it. In Table I, it can be seen that the MAS has more DNA and RNA associated with it than does the UMF. Furthermore the association of RNA with the MAS suggests that part of the membrane-enriched fraction may include microsomes.

We have also isolated a membranous fraction from cells which have been removed from the substratum by repeated CMF-PBS-EDTA-G washes. After removal, the cells are lysed in 1×10^{-3} M $ZnCl_2$, and subsequently banded in the aqueous two phase polymer system described by Brunette and Till.[94] This procedure would be expected to isolate the whole (or entire) plasma membrane (WPM) since it does not depend on the adhesion of one compartment of the membrane surface to the substratum during the isolation. The membranous fraction derived by this procedure is shown in Figure 1D. This membranous fraction appears free of gross contamination with cellular organelles. However, as has been reported by others,[94] adherent cytoplasmic material is found in this membrane-enriched isolate.

Using Na^+, K^+ ATPase as a marker enzyme for plasma membrane, we have demonstrated that the WPM fraction is approximately 15-fold enriched in plasma membrane relative to the homogenate (Table I). The data in Table I further suggest that there is limited contamination of the WPM fraction with DNA; however, the contamination with RNA is relatively high. The inclusion of RNA in this membrane-enriched fraction could result from contamination of the WPM with microsomes; however, the relatively low specific activity of glucose-6-phosphatase (Table I) (a preported marker of endoplasmic reticulum)[111,112] suggests that the majority of the RNA contamination might represent cytoplasmic RNA (e.g., free ribosomes or soluble RNA) rather than microsomal vesicles.

Using these three membrane isolation procedures, we had hoped to compare the compositions of membranes isolated from two well-defined regions of the cell (the upper and lower cell surfaces) with each other as well as with the whole plasma membrane. However, as will be shown below, the upper membrane fraction (UMF) does not represent the entire upper surface membrane of the sptr 3T3 cells. Furthermore, the fraction designated as MAS represents whole plasma membrane as well as lower plasma membrane and therefore is not an ideal membrane fraction to use in comparative studies.

Morphological Examination of Material Isolated as UMF

Figure 1A is a transmission electron micrograph (TEM) of the membranous fragment released from sptr 3T3 cells subjected to the membrane isolation procedure introduced by Barland and Schroeder.[91] Barland and Schroeder,[91] as well as Pitot and coworkers,[110] have published TEM's which demonstrated that at least a portion of the

Figure 1. Transmission electron micrographs of isolated membrane fractions and substratum attached cells after UMF isolation.

Figure 1A. Transmission electron micrograph of UMF prepared as described in Materials and Methods (x4800).

Figure 1B. Transmission electron micrograph of a sagittal section taken through cells still attached to the substratum following removal of the UMF from sptr 3T3 cells by the procedures outlined in Materials and Methods (x12,000). Cross -- cytoplasmic vesicle (see text); single arrow -- plasma membrane remaining over nucleus; double arrow -- nuclear envelope.

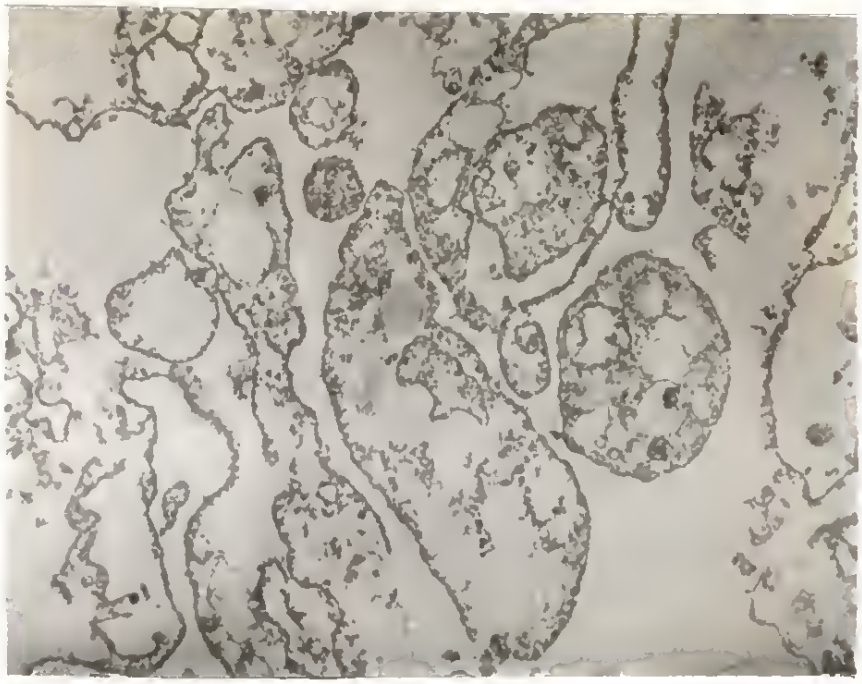


Figure 1C. Transmission electron micrograph of MAS prepared as described in Materials and Methods (x5000).

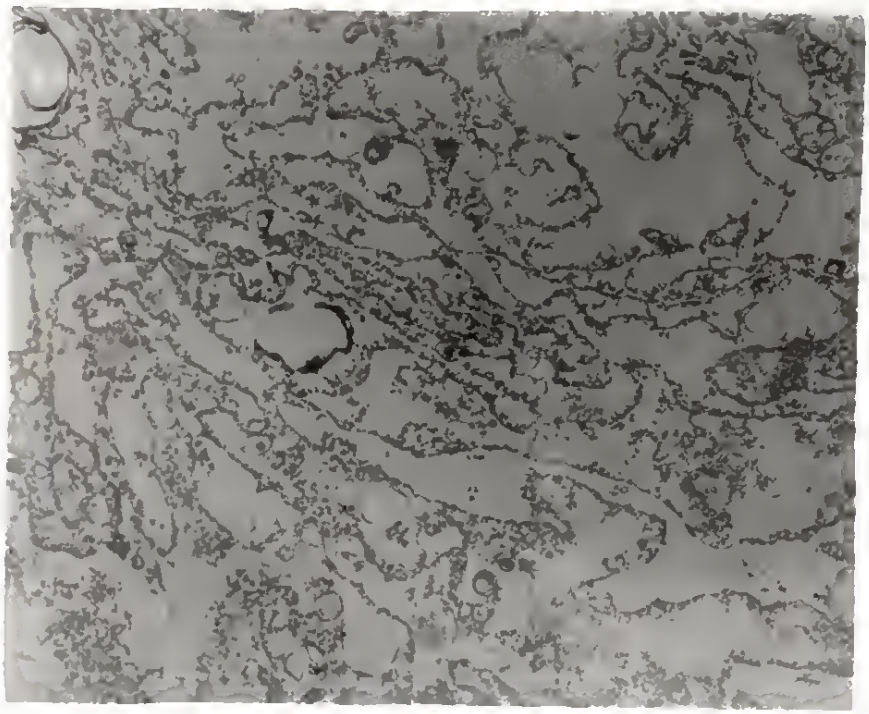


Figure 10. Transmission electron micrograph of WPM prepared as described in Materials and Methods (x5000).

upper surface of the specific cell types is released by this technique. Phase contrast micrographs demonstrate that sheets of membranous material are released from the sptr 3T3 cells used in our studies (Figure 2C) and that the majority of the cell remains attached to the substratum (compare Figures 2A and 2B) following UMF isolation. In order to better characterize both the material released by the cell and the material remaining adherent to the substratum following UMF removal, we have used scanning electron microscopy to delineate the effects of the various treatments required for the effective isolation of an UMF.

Figure 3A is a scanning electron micrograph (SEM) of sptr 3T3 cells grown to ∿80% confluency, washed with PBS, and then fixed and prepared for microscopy. As can be seen, the sptr 3T3 cells display few blebs or microfilli. Differences in gross morphology, however, can be seen between individual cells. In Figure 3A, for example, there is one cell which is very flattened and spread (epithelioid - arrow) while a neighboring cell appears spindle-shaped, having a definite long axis (fibroblastic - two arrows). As will be shown below, we have found that this morphologic diversity is of key importance to the relative efficiency of UMF isolation. In the sptr 3T3 cells used in these experiments, approximately 50% of the cells are epithelioid and approximately 50% are fibroblastic (data not shown).

The addition of $ZnCl_2$/DMSO to the sptr 3T3 cells (10 minutes, room temperature) produces a very striking increase in the number of blebs associated with the sptr 3T3 cell surface (Figures 3B and 3C). These blebs appear on all cells examined, regardless of the morphology of the individual cell. It is important to note that some of the cells

Figure 2. Phase micrographs of the sptr 3T3 cells before and after UMF isolation and the membranous material released by the UMF isolation technique.

Figure 2A. Phase micrograph of sptr 3T3 cells, untreated except for PBS wash (x600).

Figure 2B. Phase micrograph of material remaining associated with the substratum following removal of the UMF (x600).

Figure 2C. Phase micrograph of membranous material released from sptr 3T3 cells by the UMF isolation technique (x600).

Figure 3. Scanning electron micrographs of sptr 3T3 cells during the steps of the UMF isolation procedure.

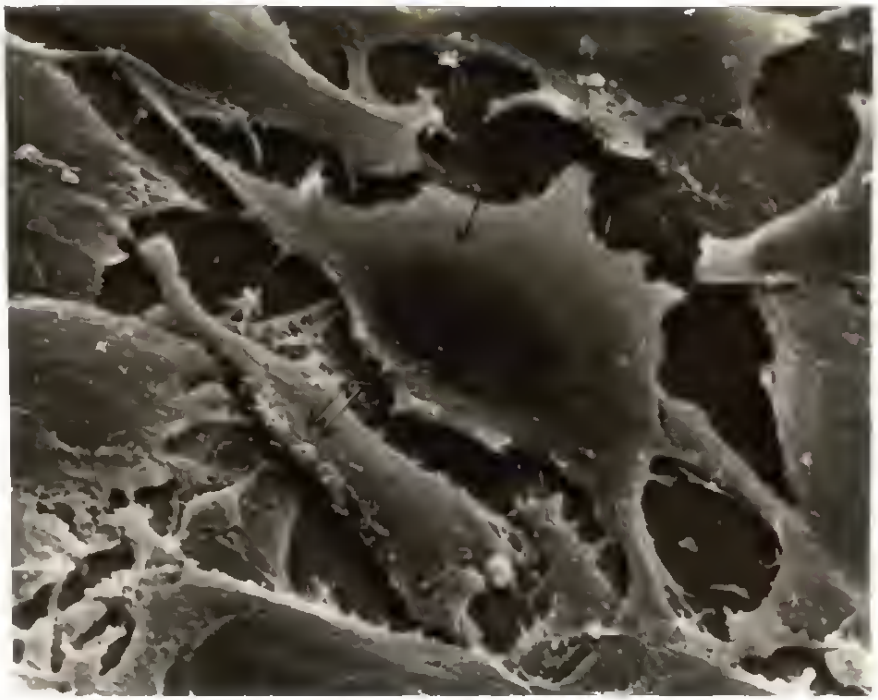


Figure 3A. Scanning electron micrograph of sptr 3T3 cells grown to 80% confluency, washed with PBS and then fixed as described in Materials and Methods (x2000). Single arrow -- sptr 3T3 cell with epithelioid morphology; double arrow -- sptr 3T3 cell with fibroblastic morphology.

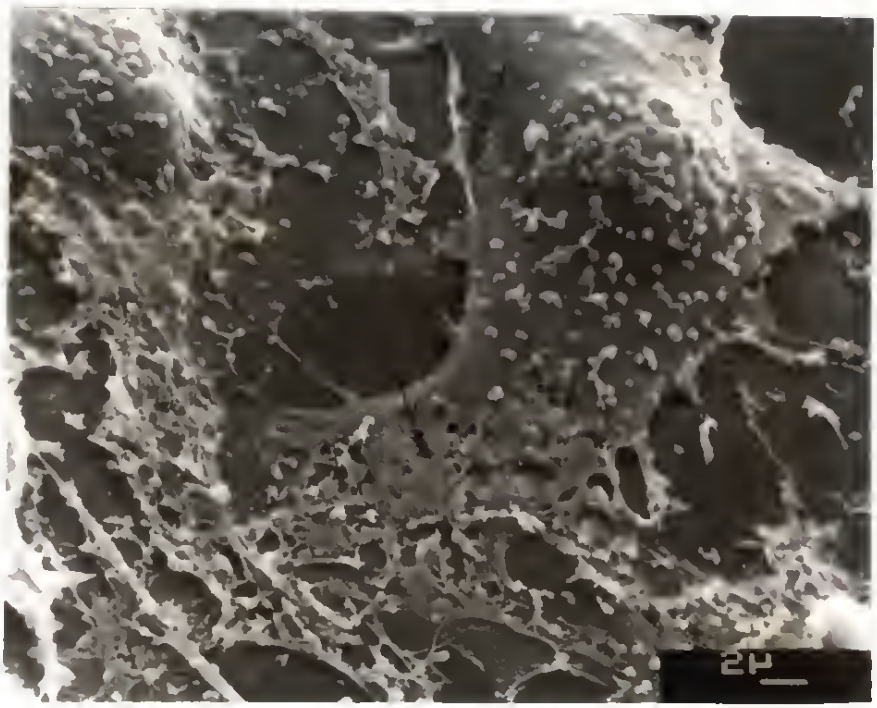


Figure 3B. Scanning electron micrograph of sptr 3T3 cells washed with PBS and then incubated 10 minutes at room temperature in $ZnCl_2$/OMSO (x3000). arrows -- holes in upper surface (see text).

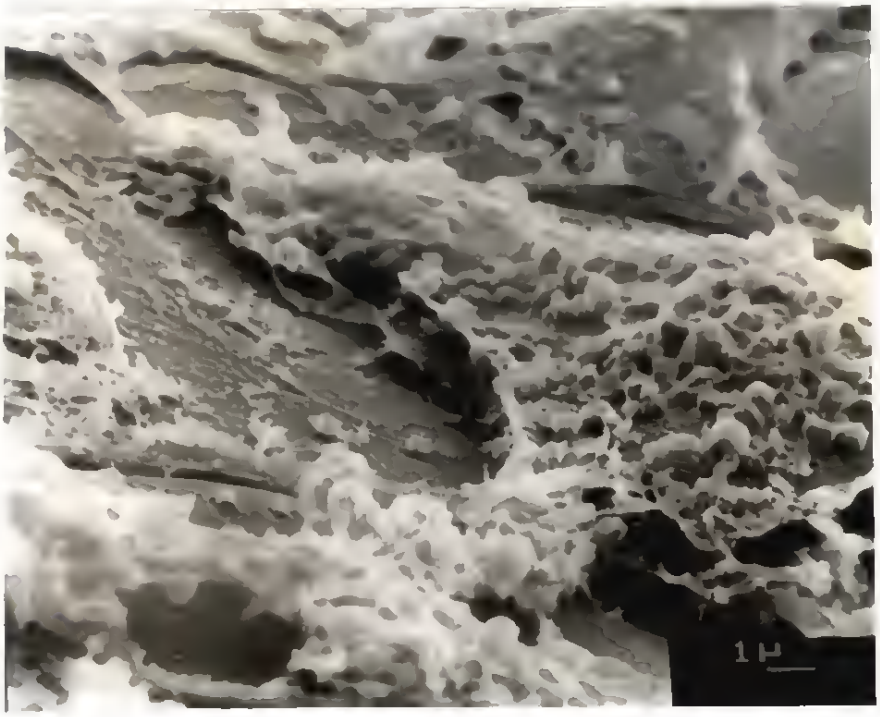


Figure 3C. Scanning electron micrograph of sptr 3T3 cells washed with PBS and then incubated 10 minutes at room temperature in $ZnCl_2$/DMSO (x6000).

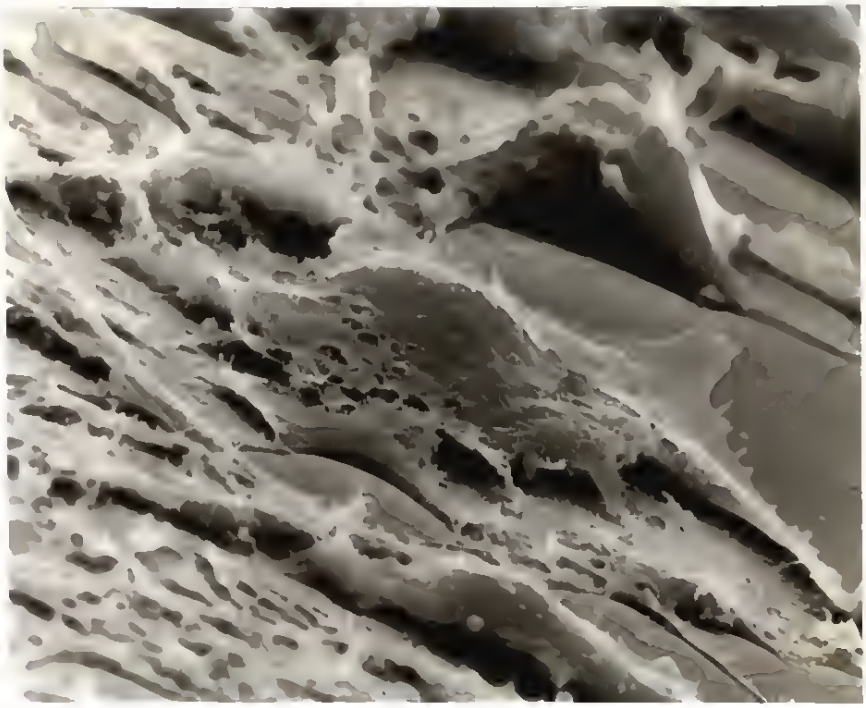


Figure 3D. Scanning electron micrograph of sptr 3T3 cells washed with PBS, incubated 10 minutes at room temperature in $ZnCl_2$/DMSO and then allowed to stand in FMA for 40 minutes at 0°C (x3000).

Figure 3E. Scanning electron micrograph of sptr 3T3 cells washed with PBS, incubated 10 minutes at room temperature in $ZnCl_2$/DMSO and then shaken for 40 minutes in FMA at 0°C (x4000). Arrow -- plasma membrane remaining at cell periphery.

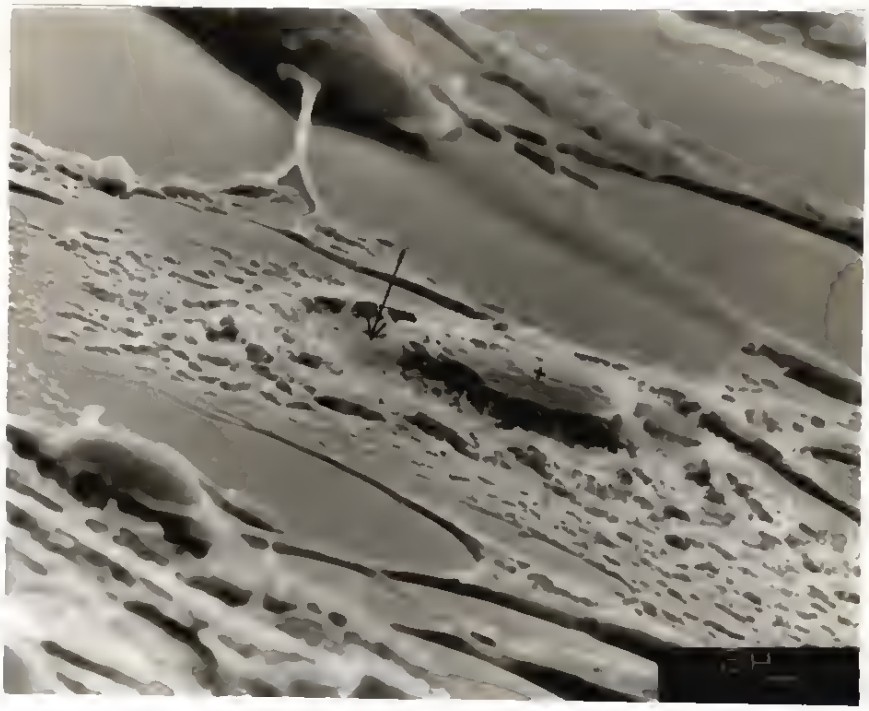


Figure 3F. Scanning electron micrograph of sptr 3T3 cells washed with PBS, incubated 10 minutes at room temperature in $ZnCl_2$/DMSO and then shaken for 40 minutes at 0°C (x2500). Arrow -- substratum-attached lower plasma membrane; cross-- membrane remaining over the nucleus.

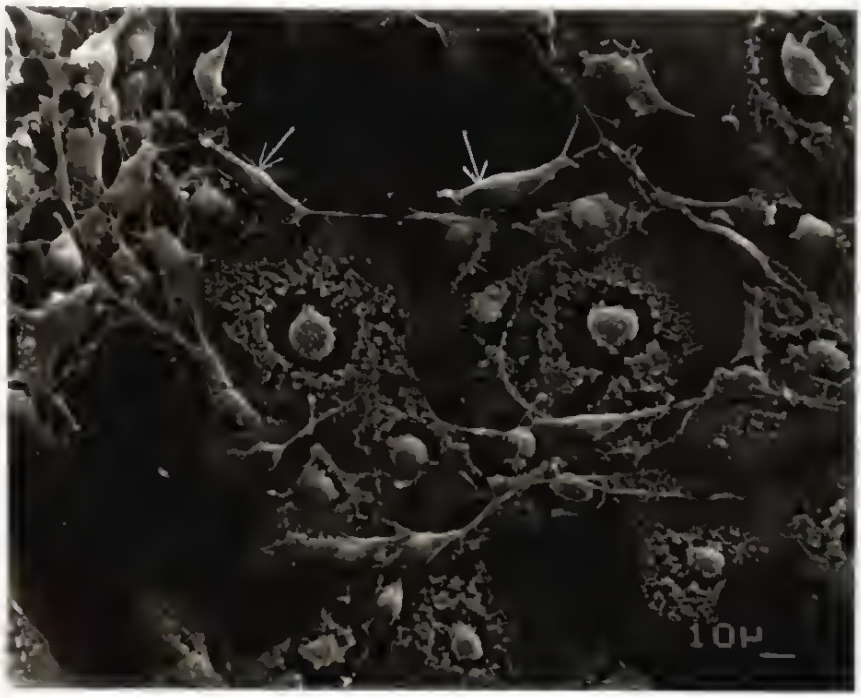


Figure 3G. Scanning electron micrograph of sptr 3T3 cells washed with PBS, incubated 10 minutes at room temperature in $ZnCl_2$/DMSO, and then shaken for 40 minutes at 0°C (x500). Arrows -- fibroblastic cells which have not released UMF.

examined after $ZnCl_2$/DMSO treatment display obvious holes in their upper surfaces (Figure 3B, arrows). Furthermore, many of the epithelioid cells treated with $ZnCl_2$/DMSO appear to contract thereby stretching the membrane at the cell edges (Figure 3B). This affect of $ZnCl_2$/DMSO treatment is observed more frequently on the epithelioid cells than on fibroblastic cells.

Incubation of the $ZnCl_2$/DMSO treated cells in FMA (40 minutes, 0°C, no shaking) clearly reduces the number of blebs on the cell surface both in fibroblastic and epithelioid cells. The FMA treatment also increases the number of holes in the upper membrane, and in some cases, appears to lift the upper membrane away from the lower membrane (Figure 3D). This increase in the number of holes is more apparent in the epithelioid cells than in the fibroblastic cells. The visible (upper) portion of the plasma membrane of the flattened epithelioid cells appears to pull inward from the substratum during the $ZnCl_2$/DMSO-FMA treatment, perhaps causing the observed holes. Although blebs occur on the fibroblastic cells with the $ZnCl_2$/DMSO treatment and disappear during the FMA treatment, contraction and extensive tearing of the plasma membrane have not been observed on these cells. Blebs which do remain on the cell after FMA treatment are only apparent over the nucleus (Figure 3G).

Rotatory shaking of the FMA treated sptr 3T3 cells (120 RPM, 40 minutes, 0°C) strips a piece of membrane from the upper surface of some, but not all, cells. The released membrane is from that portion of the upper membrane surface which circumscribes the nucleus (Figure 3E), leaving behind some membrane at the cell edges (Figure 3E, arrow), the lower, substratum-apposed membrane (Figure 3F, arrow) and a membranous "cap" situated over the nucleus (Figure 3F, cross).

It is critically important to realize that only those sptr 3T3 cells which are epithelioid in morphology release an UMF (Figure 3G). This clearly means that the membrane-enriched fraction referred to as MAS contains, at a minimum, substratum-apposed membrane (from the epithelioid cells) and whole plasma membrane (from the fibroblastic cells which do not release an UMF, Figure 3G [arrows]).

Figure 1B is a TEM of a sagittal section through the nuclear region of an sptr 3T3 cell following UMF isolation. As can be seen the plasma membrane is retained over the nucleus (arrow) and the double bilayer structure of the nuclear envelope remains intact (double arrows). Vesicles (possibly representing distended ER [crosses]) can also be seen in Figure 1B, suggesting that at least some intracellular membranes are not disrupted by the relatively harsh treatments which must precede the UMF isolation.

Peptide Composition of the UMF

The coomasie blue stained profiles of the peptides and glycopeptides of the fractions UMF, WPM, and homogenate separated via SDS-PAGE are presented in Figure 4. Although many of the coomasie blue staining bands of the UMF and WPM are clearly running with the same MW's, it is evident from the profiles displayed in Figure 4 that there are compositional differences between the UMF and WPM which can be detected by SDS-PAGE. These differences are most obvious among the high molecular weight (MW $> \sim$120,000) peptides and glycopeptides which are much less prominent in the coomasie blue stained profile derived from UMF as compared to the profile obtained from the WPM.

Extraction of Membrane Components by $ZnCl_2$/DMSO-FMA

One possible explanation for the compositional differences displayed in Figure 4 would be that the $ZnCl_2$/DMSO-FMA pretreatment of

Figure 4. Electrophoretic profile of UMF, MAS, and a whole cell homogenate.

Coomasie blue staining profile of UMF, WPM, and whole cell homogenate peptides and glycopeptides separated on a 7.5-12.5% linear SDS-PAGE. UMF and WPM were isolated from sptr 3T3 cells, solubilized in Laemmli sample buffer[97] and separated via electrophoresis. From left to right: molecular weight markers (phosphorylase a, 100,000; bovine serum albumin, 67,000; ovalbumin, 43,000; deoxyribonuclease I, 31,000; soybean trypsin inhibitor, 21,500; cytochrome c, 12,400); Lane 1, UMF; lane 2, WPM; lane 3, homogenate of whole cells. Fifty micrograms of protein were applied to each line. The homogenate was digested with 75 µg/ml each deoxyribonuclease I and ribonuclease A (20 minutes, room temperature) prior to solubilization.

the sptr 3T3 cells specifically extracts those components of the cell membrane missing from the UMF profile. In early work relating to this potential problem, we demonstrated that the coomasie blue staining profile of WPM's isolated from sptr 3T3 cells treated with $ZnCl_2$/DMSO-FMA prior to membrane isolation via the technique of Brunette and Till[94] was the same as the WPM profile obtained from sptr 3T3 cells isolated without pretreatment with the heavy metals (Figure 5). Although these data suggested that no preferential and total extraction of specific membrane components resulted from the $ZnCl_2$/DMSO-FMA pretreatments, the data could not rule out a quantitative, as opposed to qualitative, extraction of membrane proteins or glycoproteins. To rule out such a quantitative extraction of membrane components via the $ZnCl_2$/DMSO-FMA pretreatment, cells were radiolabeled with [^{3}H] leucine, [^{3}H] glucosamine, or [^{3}H] choline and the relative extraction of membrane peptides, glycopeptides, or phospholipids determined.

Data relating to the possible extraction of labeled material from substratum-attached cells by the $ZnCl_2$/DMSO-FMA treatments are displayed in Table II. The non-dialyzable leucine counts extracted into the $ZnCl_2$/DMSO wash amounted to only D.1% of the total cell counts, whereas 21% of the total label was extracted into FMA (40 minutes, 0°C, no shaking). Figure 6 is a fluorograph of the extracted, non-dialyzable leucine-labeled material. This fluorograph demonstrates that the material which was extracted by the FMA did not co-migrate with components which were missing from the UMF (Figure 4) suggesting that extraction could not account for the peptides missing from the UMF fraction relative to the WPM (see Figure 4). $ZnCl_2$/DMSO extraction of [^{3}H] glucosamine labeled cells removed D.2% of the total label into

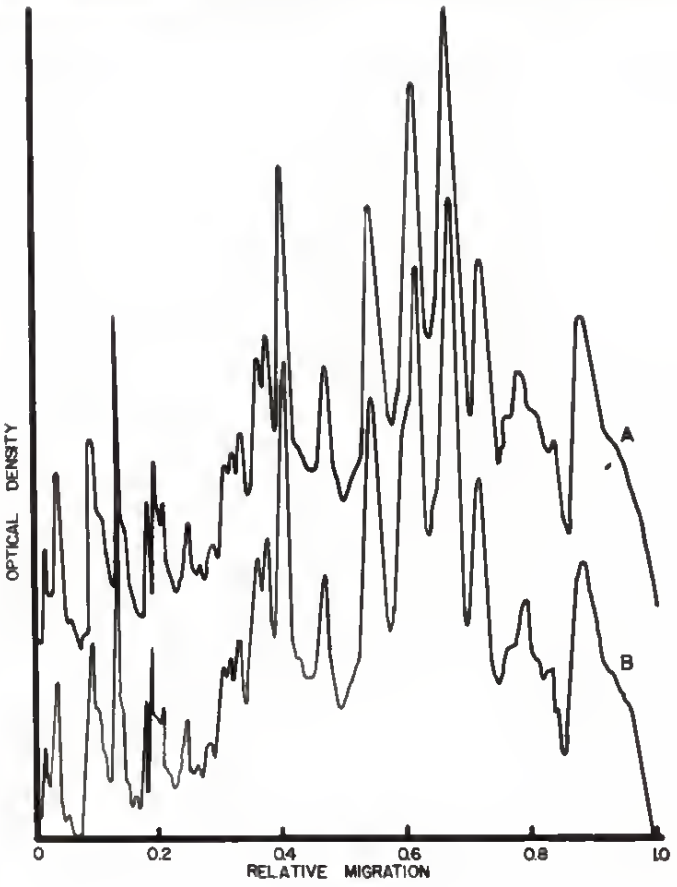


Figure 5. Effects of $ZnCl_2$/OMSO-FMA on WPM composition.

Densitometric scan of WPM peptides and glycopeptides separated on an 8% SOS-PAGE tube gel, then stained with coomasie blue.

A. WPM from untreated sptr 3T3 cells

B. WPM isolated from sptr 3T3 cells which had been preincubated with $ZnCl_2$/OMSO (10 minutes room temperature) followed by FMA (40 minutes, 0°C) prior to isolation by the technique of Brunette and Till.[94]

Table II

Extraction of Labeled Material from Whole Cells

Radioactive Precursor	Treatment: PBS Wash % of whole cells		Treatment: $ZnCl_2$/DMSO % of whole cells		Treatment: FMA % of whole cells	
	pre-dialysis	post dialysis	pre-dialysis	post dialysis	pre-dialysis	post dialysis
[^{3}H] leucine	0.4%	0.1%	2%	0.1%	29%	21%
[^{3}H] glucosamine	0.5%	0.1%	6%	0.2%	29%	7%
[^{3}H] choline	0.9%	N.D.	14%	N.D.	16%	N.O.

Cells were grown for 72 hours in the presence of the indicated precursors. The cells were then washed 5 times with PBS. The number of counts released into the fifth wash was determined. Following the PBS wash the same cells were incubated for 10 minutes at 22°C with $ZnCl_2$/OMSO and the counts released determined. Finally the same cells were incubated 40 minutes at 0°C (without shaking) in FMA and the counts released determined. The released leucine and glucosamine labeled material was dialyzed in an effort to remove any free radioisotope which was released from soluble pools. The material released from choline labeled cells was not dialyzed for fear of losing small lipid micelles along with any free choline pool. N.O.: not determined.

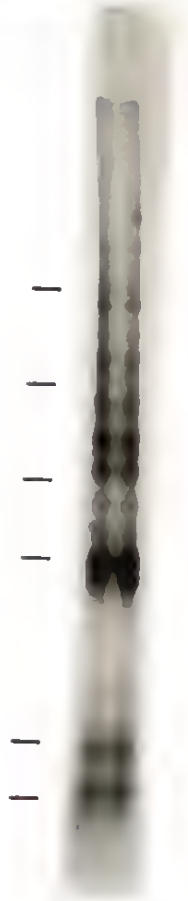


Figure 6. [^{3}H] Leucine containing material extracted by FMA. Fluorograph of non-dialyzable, [^{3}H] leucine labeled material extracted into FMA from sptr 3T3 cells treated with $ZnCl_2$/DMSO (10 minutes, room temperature) and FMA (40 minutes; 0°C). The material was solubilized in Laemmli sample buffer[97] and components separated on a 7.5-12.5% linear SDS-PAGE. Following separation, the [^{3}H] leucine labeled components were visualized by the fluorographic technique of Bonner and Laskey.[99] Approximately 5 x 10^4 cpm's were applied to the gel. From left to right: markers corresponding to the molecular weight standards shown in Figure 4, [^{3}H] leucine labeled material.

non-dialyzable material while FMA treatment extracted 7% of the total cell glucosamine as non-dialyzable material. Fluorography of the extracted, [^{3}H] glucosamine-labeled material also failed to demonstrate a preferential extraction of glycopeptides which co-migrated with the membranous components missing from the UMF (data not shown). $ZnCl_2$/DMSO extraction of [^{3}H] choline-labeled whole cells removed 14% of the total cell counts while FMA treatment extracted another 16% of the [^{3}H] choline counts. Although it was not feasible to dialyze the [^{3}H] choline labeled material, it was virtually certain that some (if not all) of the extracted choline label represented material taken from the soluble pool of the cell.

The data presented in Table II could be taken to indicate actual extraction of membrane components. However the "extracted" material could also represent the leakage of soluble intracellular pools and cytoplasmic components through the holes in the membrane produced by $ZnCl_2$/DMSO and FMA pretreatments of the sptr 3T3 cells (see Figures 3B-3G). To distinguish between these alternatives, radiolabeled WPM's were isolated via the Brunette and Till[94] technique and then treated sequentially with $ZnCl_2$/DMSO and FMA. Table III contains the accumulated data. Treatment of [^{3}H] leucine labeled membranes with PBS (PBS_1, 10 minutes, room temperature) removed 1.7% of the total radiolabel associated with the membrane while treatment with $ZnCl_2$/OMSO (10 minutes, room temperature) removed 2.5% of the label. Further treatment of the same membranes with PBS (PBS_2, 40 minutes, 0°C) of the $ZnCl_2$/OMSO "tanned" membranes removed 3.1% of the counts while FMA treatment (40 minutes, 0°C) removed 3.9% of the counts. Treatment of [^{3}H] glucosamine labeled WPM's with PBS (PBS_1, 10 minutes,

Table III

Extraction of Labeled Material from Isolated WPM

Treatment	Radioactive Precursor		
	[^{3}H] leucine % of WPM	[^{3}H] glucosamine % of WPM	[^{3}H] choline % of WPM
PBS_1	1.7%	6.1%	1.1%
$ZnCl_2$/OMSO	2.5%	2.3%	2.4%
PBS_2	3.1%	2.3%	0.6%
FMA	3.9%	3.8%	2.2%

Cells were grown for 72 hours in the presence of the labeled precursor. The WPM fraction was then isolated according to the technique of Brunette and Till.[94] The membranes were all washed once with PBS and then incubated 10 minutes at 22°C in PBS (PBS_1) or $ZnCl_2$/OMSO. The membranes were pelleted and the percent extracted material determined. The pellet was resuspended in PBS (PBS_2) or FMA and incubated 40 minutes at 0°C. The membranes were again pelleted and the percent material extracted determined.

room temperature) removed 6.1% of the label while treatment with $ZnCl_2$/OMSO (10 minutes, room temperature) removed only 2.3% of the counts. A second incubation in PBS (PBS_2, 40 minutes, 0°C) removed another 2.3% of the total glucosamine label while FMA treatment of the $ZnCl_2$/DMSO fixed membranes removed 3.8% of the counts. Treatment of [^{3}H] choline labeled WPM's with PBS (PBS_1, 10 minutes, room temperature) removed 1.1% of the total membrane associated counts while $ZnCl_2$/OMSO (10 minutes, room temperature) removed 2.4% of the counts. A second PBS wash (PBS_2, 40 minutes, 0°C) removed another 0.6% of the [^{3}H] choline while FMA treatment (40 minutes, 0°C) removed 2.2% of the total label. Thus in each instance, $ZnCl_2$/DMSO plus FMA treatment removed 5-6% of the total label associated with the membrane while sequential incubations in PBS removed 3-7% of the total labeled material. These data suggest that neither the $ZnCl_2$/DMSO nor the FMA pretreatment of the membrane extracts significant quantities of membranous material relative to the amount of membranous material extracted by sequential PBS washes.

It could be argued that loss of labeled membrane components from the cells attached to the substratum results from the release of membranous blebs into the $ZnCl_2$/DMSO or FMA solutions (see Figures 3C and 3D). If this were indeed the case, one might have expected that the membrane remaining associated with the substratum following release of the UMF would display a SDS-PAGE profile similar to that shown for the UMF (Figure 4). In order to test this possibility, a membrane fraction was isolated from the material remaining attached to the substratum (the MAS) via the technique outlined in the Materials and Methods section (see Figure 1C for a TEM of the isolated

material). Figure 7 demonstrates that the components missing from the UMF profile were present in exaggerated amounts in the MAS (relative to the WPM) and that mixing equal quantities of UMF and MAS produced a coomasie blue staining profile very similar to the WPM.

Density and Lipid Composition of UMF and WPM

One of the earliest findings with regard to the properties of the UMF was the apparent high density of the UMF. Specifically, when UMF was resuspended in 55% (w/w) sucrose, overlayered with a linear sucrose gradient ranging from 22-50% (w/w) sucrose and centrifuged at ∿110,000 x g for 18 hours, the UMF remained in the 55% sucrose cushion (1.2619g/ml). There was no evidence that any membranous material floated out of this very dense sucrose. MAS (isolated from the same cells which had yielded the UMF) when resuspended in 55% (w/w) sucrose, overlayered with a 22-50% (w/w) sucrose gradient, and centrifuged for 18 hours at 110,000 x g, floated up into a band at approximately 45% (w/w) sucrose (1.2090g/ml). WPM, isolated via the technique of Brunette and Till,[94] when resuspended in 55% (w/w) sucrose, overlayered with a 23-53% (w/w) linear sucrose gradient and centrifuged for 18 hours at 110,000 x g, spun up into a tight band at approximately 36% (w/w) sucrose (1.1566g/ml).

In order to determine whether or not the high density of the UMF might be related to an altered protein to lipid ratio, the UMF and WPM samples were extracted with chloroform-methanol[104] and the amount of phospholipid extracted was determined. These analyses yielded values of 15.6µg phospholipid per mg protein for the UMF and 275µg phospholipid per mg protein for the WPM, thereby suggesting a 17-fold reduction in phospholipid in the UMF relative to the WPM.

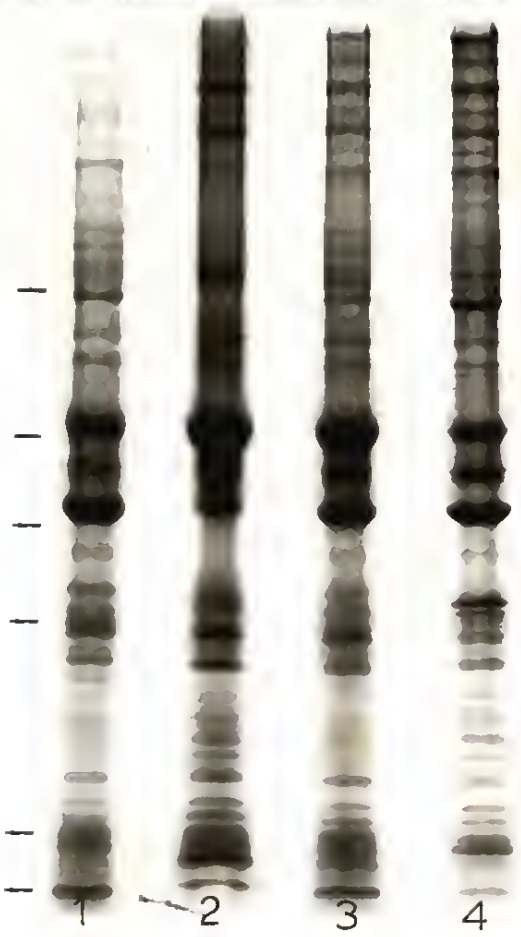


Figure 7. Electrophoretic profile comparing UMF, MAS, WPM, and UMF plus MAS.

Coomasie blue staining profile of membrane fractions separated in a 7.5-12.5% SDS-PAGE after isolation from sptr 3T3 cells and solubilized with Laemmli buffer.[97] From left to right: markers corresponding to molecular weight standards shown in Figure 4; Lane 1, UMF; lane 2, MAS; lane 3, WPM; lane 4, UMF plus MAS. Fifty micrograms of protein were applied to each well. The UMF-MAS mixture (lane 4) contained 25 µg UMF plus 25 µg MAS.

Since other data (see Table III) suggested that very little, if any, phospholipid was extracted from the plasma membrane by the $ZnCl_2$/DMSO or FMA treatment during the isolation procedure, we considered it possible that the apparently low phospholipid content of the UMF resulted from a reduced solubility of the phospholipid in chloroform-methanol following $ZnCl_2$/DMSO or FMA treatment of the cells. In order to circumvent this possible problem, the sptr 3T3 cells were maintained for 72 hours in medium supplemented with labeled precursors of membrane lipids (Table IV). Membrane isolates were then prepared and the specific activities of each membrane isolate compared. As can be seen in Table IV, with the precursors used, the WPM always displayed a significantly higher specific activity than did the UMF. When MAS was isolated from labeled cells (Table IV), the specific activity of the MAS was higher than either the UMF or WPM.

These data clearly indicated that the UMF represented a protein rich, lipid depleted fraction of the plasma membrane. Although we had demonstrated that the $ZnCl_2$/DMSO-FMA treatments extracted very little phospholipid or protein from isolated WPM, we nevertheless decided to investigate the possibility that the $ZnCl_2$/DMSO-FMA treatments act on intact cells to release lipid-rich vesicles which might contain the high molecular weight peptides missing from the UMF. In these experiments sptr 3T3 cells were grown for 72 hours in 2.5μCi/ml [^{32}P] orthophosphate to label the phospholipids. Cells were then washed with 1.6×10^{-1}M NaCl/6.8×10^{-7}M $CaCl_2$, followed by incubation in $ZnCl_2$/DMSO (10 minutes, room temperature) and then FMA (40 minutes, 0°C, no shaking). The supernatant from each treatment was brought to 55% (w/w) sucrose and overlayered with a 10%-35% (w/w) linear sucrose

Table IV

Relative Lipid Precursor Incorporation into UMF, MAS, and WPM

Radioactive Precursor	CPM's/μg Protein UMF	MAS	WPM	Ratio WPM/UMF	Ratio MAS/UMF
[^{14}C] glycerol	73	1081	486	6.5/1 (n=5)	14.5/1 (n=2)
[^{3}H] choline	475	14667	10120	20.1/1 (n=3)	27.5/1 (n=2)
[^{3}H] palmitate	1632	15267	13282	8.0/1 (n=2)	9.2/1 (n=2)
[^{3}H] ethanolamine	514	N.D.	4842	9.4/1 (n=1)	N.O.
[^{3}H] inositol	6	N.D.	29	4.9/1 (n=1)	N.O.

Sptr 3T3 cells were maintained for 72 hours in medium containing the indicated concentration of radiolabeled precursor (see Materials and Methods). The three membrane fractions were then isolated and the incorporation of each precursor into the fractions determined. The incorporation into each fraction is taken from a representative experiment while the ratios are mean values derived from the number of individual experiments indicated in the parentheses. N.O.: not determined; n = number of times each precursor was used.

gradient. This gradient was centrifuged 4 hours at 48,000 x g and then fractionated. One would predict that any lipid-rich vesicles which had been released during any of the three washings would move out of the high density sucrose cushion and band (or at least disperse) somewhere in the linear portion of the sucrose gradient. As can be seen in Figures 8A-8C, there was no evidence for the movement of any [^{32}P] labeled components into the low density regions of the sucrose gradient, suggesting that few, if any, lipid-rich vesicles were extracted during the PBS washes or the $ZnCl_2$/DMSO or FMA incubations which preceded the UMF isolation procedure.

Fixation of Extraneous Material to the UMF

A number of experiments were designed to investigate whether or not the UMF represented a metabolic product of the cell or was composed primarily of extraneous adherent material. In order to demonstrate that the UMF was composed of components synthesized by the cell, the sptr 3T3 cells were grown for 72 hours in 2.5μCi/ml [^{3}H] leucine, the UMF isolated, and membrane peptides separated via SDS-PAGE. Figure 9 is a fluorograph of UMF radiolabeled with [^{3}H] leucine. This fluorograph demonstrates that all major coomasie blue staining bands (see Figure 4) were labeled, suggesting that the UMF components were products of cellular metabolism rather than a $ZnCl_2$/DMSO-FMA induced aggregation of components from the medium.

In experiments designed to determine whether this UMF was composed primarily of surface coat material made up of sulfated glycosaminoglycans or collagen, sptr 3T3 cells were metabolically labeled with either 100μCi/ml [^{35}S] or 5μCi/ml [^{3}H] proline and the UMF and WPM isolated. As Table V shows, there was a moderate increase in [^{35}S]

Figure 8. Density separation of [^{32}P] containing material released by $ZnCl_2$/OMSO and FMA treatments.

Profile of three sucrose gradients intended to display any lipid rich vesicles released from the sptr 3T3 cells treated as described in the text. These gradients were centrifuged at 110,000 x g for 4 hours.

A. Cells were washed with 1.6×10^{-1}M NaCl/ 9×10^{-3}M $CaCl_2$. An aliquot from this wash was then brought to 55% (w/w) sucrose and overlayered with a 10-35%(w/w) linear sucrose gradient.

○: cpm's [^{32}P]/10 µl sample

B. After the NaCl/$CaCl_2$ wash, the cells were incubated in $ZnCl_2$/OMSO (10 minutes, room temperature). An aliquot of this $ZnCl_2$/OMSO solution was then brought to 55% (w/w) sucrose and overlayered with a 10-35% (w/w) linear sucrose gradient.

⊙: cpm's [^{32}P]/10 µl sample

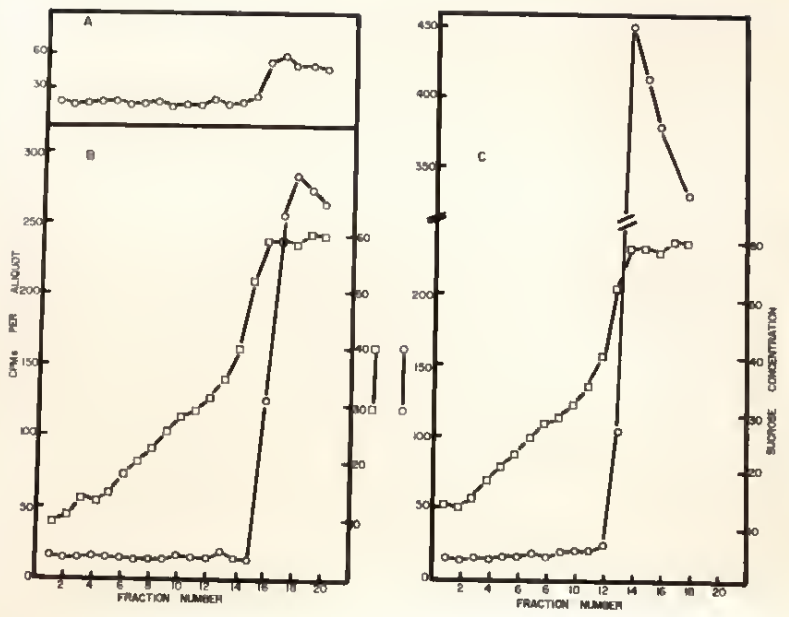
□: sucrose concentration (from refractive index)

C. After $ZnCl_2$/OMSO treatment, the cells were incubated in FMA (40 minutes, 0°C). An aliquot of the FMA solution was then brought to 55% (w/w) sucrose and overlayered with a 10-35% (w/w) linear sucrose gradient.

○: cpm's [^{32}P]/10 µl sample

□: sucrose concentration (from refractive index)

Each fraction from the gradient was 2.0 mls. The sucrose density gradient was the same in both A and B.


A
B
C
CPMs PER ALIQUOT
SUCROSE CONCENTRATION
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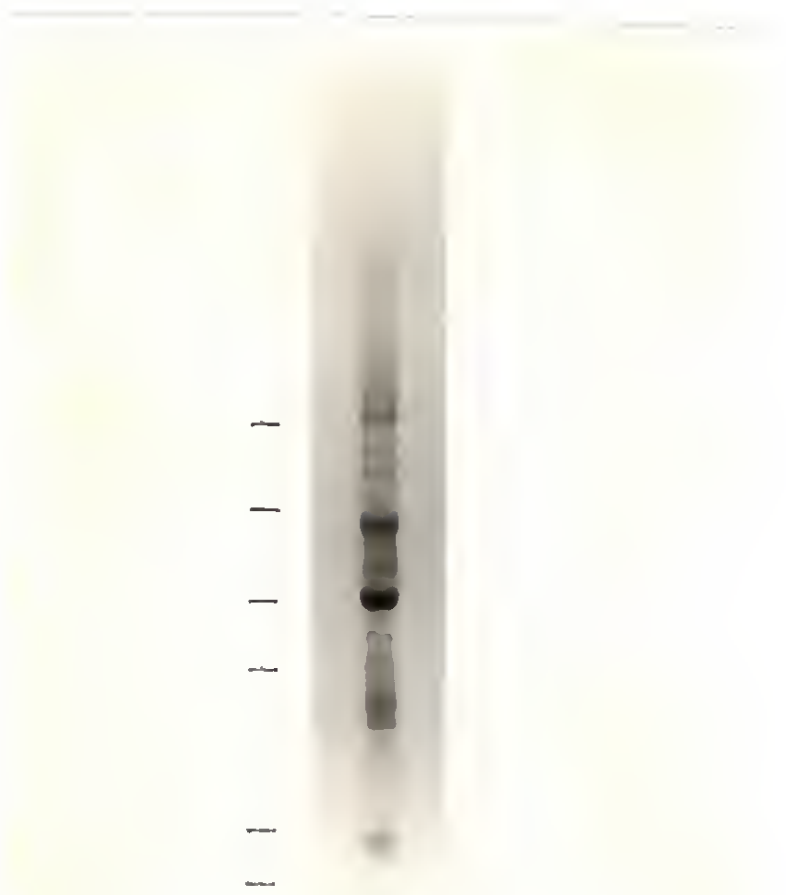


Figure 9. [^{3}H] Leucine containing components of the UMF
Fluorograph of UMF peptides and glycopeptides isolates from sptr 3T3 cells grown for 72 hours in medium containing 2.5 μCi/ml [^{3}H] leucine. After isolation, the membrane fraction was solubilized in Laemmli sample buffer[97] and components separated on a 7.5-12.5% SDS-PAGE. Following separation the labeled components were displayed by the fluorographic technique of Bonner and Laskey.[99] Approximately 1 x 10^5 cpm's were applied to the gel. From left to right: markers corresponding to the molecular weight standards shown in Figure 4; [^{3}H] leucine labeled UMF.

Table V

Relative Precursor Incorporation into UMF and WPM

Radioactive Precursor	CPM's/µg Protein UMF	WPM	Ratio WPM/UMF
[^{3}H] glucosamine	94	2141	20.7/1 (n=10)
[^{3}H] fucose	16	389	20.3/1 (n=4)
[^{35}S]	9	13	1.4/1 (n=2)
[^{3}H] proline	5946	4546	0.77/1 (n=1)

Sptr 3T3 cells were maintained for 72 hours in medium containing the indicated concentrations of radiolabeled precursors (see Materials and Methods). Following growth the two membrane fractions were isolated and the CPM's/µg protein determined for each precursor. Although the UMF and WPM fractions are clearly handled differently during the isolation procedures, we believe the relative values displayed as ratios of WPM/UMF are valid since all of our work suggests that $ZnCl_2$/FMA treatment does not extract membrane components. The numbers in the parentheses associated with the ratio WPM/UMF indicate the number of times each experiment was performed. The CPM's/µg protein for UMF and WPM are taken from one representative experiment while the ratio is a mean of the various experiments done with each precursor.

specific activity in the WPM relative to the UMF, clearly demonstrating that this UMF was not composed predominantly of sulfated glycosaminoglycans.

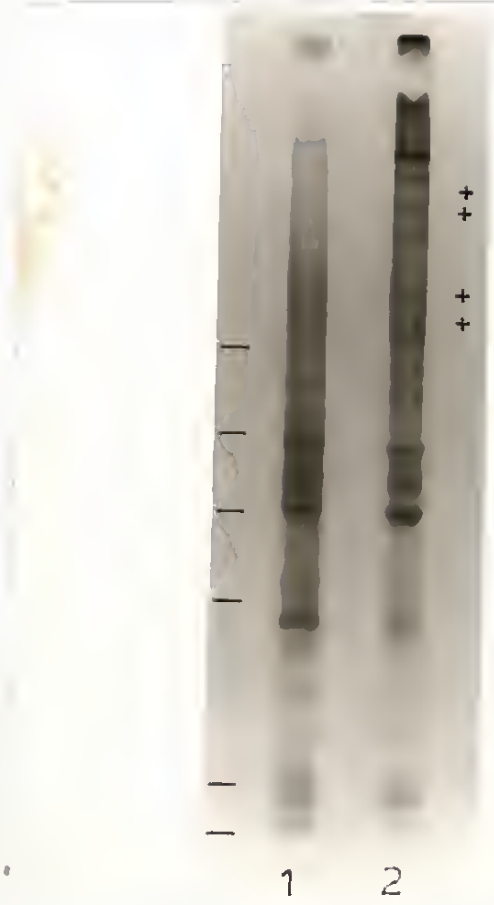
Figure 10 is a fluorograph of UMF and WPM isolated from sptr 3T3 cells maintained for 72 hours in 5μCi/ml [^{3}H] proline. When this fluorograph is compared with the coomasie blue staining profile of UMF and WPM presented in Figure 4, it is apparent that virtually all of the major membrane components of the two membrane isolates were labeled with [^{3}H] proline. More importantly, there is no evidence from this fluorograph for a preferential labeling of components migrating at the same apparent molecular weights as the various species derived from a rat tail collagen standard (crosses) which was co-electrophoresed with the proline labeled membrane fractions. Furthermore, Table V demonstrates that the specific activity of the [^{3}H] proline labeled UMF was only moderately higher (1.3 fold) than the specific activity of the WPM, again suggesting that the $ZnCl_2$/DMSO-FMA isolation technique did not preferentially isolate a collagenous surface coat.

As would be expected from the isotope incorporation data, collagenase digestion (20μg/ml, 10 minutes at room temperature) of the sptr 3T3 cells prior to either UMF or WPM isolation did not change the coomasie blue staining SDS-PAGE profile derived from either membrane fraction (data not shown).

It was clear to us that the possibility existed that significant quantities of serum proteins might become adherent to the UMF during the $ZnCl_2$/DMSO-FMA treatment. This possibility was tested using cells grown for 72 hours in serum radiolabeled with [^{125}I]. The specific activity of the UMF isolated from these cells demonstrated that calf

Figure 10. [^{3}H] Proline containing components of UMF and WPM

Fluorograph of [^{3}H] proline labeled membrane fractions. The sptr 3T3 cells were maintained for 72 hours in medium containing 4.0 μCi/ml [^{3}H] proline. The UMF and WPM fractions were isolated, solubilized in Laemmli sample buffer[97] and components separated on a 7.5-12.5% SDS-PAGE. Approximately 3 x 10^5 cpm's were applied to each well of the gel. Following separation the labeled components were displayed by the fluorographic technique of Bonner and Laskey.[99] From left to right: markers corresponding to molecular weight standards shown in Figure 4; Lane 1, UMF; lane 2, WPM; markers (crosses) corresponding to unlabeled rat tail collagen standards which were coelectrophoresed with the membrane fractions (MW's: 175,000; 150,000; 120,000; 110,000). No exogenous ascorbic acid was added to the sptr 3T3 cells since we found that even at low concentrations, ascorbic acid was toxic to sptr 3T3 cells. The toxicity of exogenously added ascorbic acid has been reported in other systems[113] and it has been previously reported that transformed cells can synthesize collagen in the absence of exogenous ascorbic acid.[114]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serum components could, at most, represent 1µg per mg of the UMF isolate, suggesting that adherent serum peptides were very unlikely to be responsible for any of the major coomasie blue staining bands of the UMF.

Iodinateable Surface Components

Membrane peptides and glycopeptides of the sptr 3T3 cells were iodinated via the technique of Phillips and Morrison.[100] The relative differences in iodinateable surface components associated with the UMF as compared to the WPM and MAS are expressed in Table VI. These data indicate that the specific activity of iodinateable surface components was approximately 3.2 times greater in the WPM relative to the UMF and 13 times greater in the MAS relative to the UMF. These data clearly suggest that the majority of the iodinateable surface components were associated with that area of the cell surface apposed to the substratum.

Figure 11 is an autoradiograph of iodinated peptides and glycopeptides of the UMF and WPM separated on SDS-PAGE. As can be seen, the WPM fraction presented a complex pattern of iodinateable surface components, many of which were either not found in the UMF or were present in such low amounts that they could not be detected by autoradiography. Interestingly, the MAS contained all the iodinated surface components found in the WPM (data not shown).

It is worth noting, with regard to specific surface components, that in the sptr 3T3 cell culture used, the WPM apparently contained more LETS[28,29](cross) per mg protein than did the UMF. Evidence from a variety of other laboratories[30-33] would support a localization of LETS to the cell periphery or the underside of the cell.

Table VI

Iodination of Membrane Components

CPM's/µg Protein			Ratio	Ratio
UMF	WPM	MAS	WPM/UMF	MAS/UMF
340	1420	4320	3.2 (n=5)	13.2 (n=4)

Sptr 3T3 cells were iodinated, while still attached to the substratum, according to the technique described by Phillips and Morrison.[100] Following iodination the various membrane fractions were isolated. The CPM's/µg protein for UMF, WPM, and MAS are taken from one representative experiment while the ratios are means of the various experiments performed. n = number of times each experiment was performed.

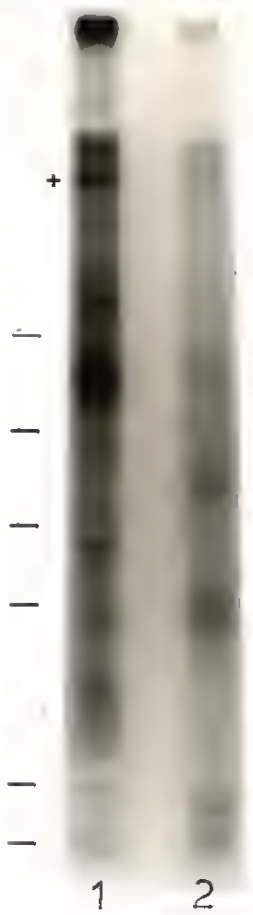

Figure 11. [^{125}I] Labeled components of UMF and WPM
Autoradiograph of [^{125}I] labeled UMF and WPM. Using the technique of Phillips and Morrison,[100] sptr 3T3 cells were labeled with [^{125}I] while still attached to the substratum. Following iodination, the UMF and WPM were isolated, solubilized in Laemmli sample buffer[97] and separated on a 7.5-12.5% SDS-PAGE. Approximately 2 x 10^4 cpm's were applied to each well of the gel. After separation, the iodinated species were displayed by autoradiography. From left to right: markers corresponding to molecular weight standards shown in Figure 4; Lane 1, WPM; lane 2, UMF. Cross -- LETS protein (see text).

Thus both the relative specific activities of the iodinated membrane fractions as well as the autoradiographic profile obtained after peptide and glycopeptide separation on SDS-PAGE suggest that, in the sptr 3T3 cells used in these studies, the majority of iodinateable surface components were associated with areas of the plasma membrane other than that represented by the UMF.

Glycoproteins of the UMF and WPM

A fluorograph of UMF, WPM, and MAS fractions isolated from stpr 3T3 cells maintained in medium containing 2.5μCi/ml [^{3}H] glucosamine is presented in Figure 12. Only two major glucosamine-labeled bands plus a number of minor bands were detected in the UMF sample while approximately 20 labeled glycopeptides could be identified in both the WPM and MAS sample. As was the case with iodinated surface components, the fluorographic profile of MAS isolated from the cells maintained in medium containing [^{3}H] glucosamine was essentially identical to the profile obtained for WPM. Virtually the same fluorographic profiles were obtained when the three membrane fractions were isolated from cells maintained in medium supplemented with 1.0μCi/ml [^{3}H] fucose. [^{3}H] fucose has the advantage, in experiments of this type, of being preferentially incorporated into the glycosyl residues of glycoproteins but not glycosaminoglycans or mucopolysaccharides.[115] Thus, [^{3}H] fucose is unlikely to label surface coat material and lends further support to our content that the UMF is a true membrane fraction.

In related experiments, sptr 3T3 cells were labeled with either $NaIO_4/NaB[^3H]_4$ (to label glycoproteins or glycolipids which contain sialic acid) or galactose oxidase/$NaB[^3H]_4$ (to label glycoproteins or

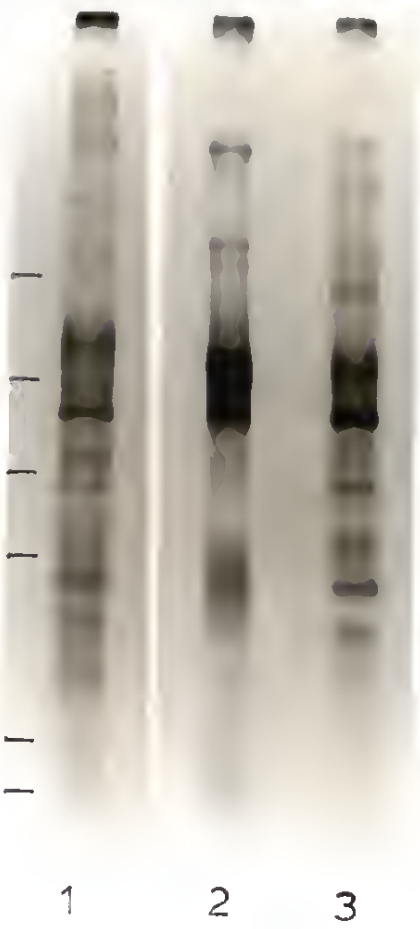

Figure 12. [^{3}H] Glucosamine containing components of UMF, MAS, and WPM

Fluorograph of [^{3}H] glucosamine labeled UMF, MAS, and WPM. The sptr 3T3 cells were maintained for 72 hours in medium containing 2.5 µCi/ml [^{3}H] glucosamine. The three membrane fractions were then isolated, solubilized in Laemmli sample buffer[97] and separated on a 7.5-12.5% SDS-PAGE. Approximately 1 x 10^5 cpm's were applied to each well of the gel. The [^{3}H] glucosamine containing components were identified by the fluorographic technique of Bonner and Laskey.[99] From left to right: markers corresponding to molecular weight standards shown in Figure 4; Lane 1, WPM; lane 2, UMF; lane 3, MAS.

glycolipids containing galactose residues). Fluorographs prepared from the three membrane fractions demonstrated that while the WPM and MAS displayed approximately 20 glycopeptides, the UMF displayed only 2 major bands and a few minor bands (data not shown).

Table V gives the specific activity of UMF and WPM isolated from sptr 3T3 cells maintained for 72 hours either in 2.5μC/ml [^{3}H] glucosamine or 1.0μC/ml [^{3}H] fucose. The specific activity of [^{3}H] glucosamine labeled WPM is approximately 20 times higher than the specific activity of the UMF isolated from cells maintained under the same growth conditions. Similarly, the specific activity of WPM isolated from [^{3}H] fucose labeled cells is 20 times higher than that of the UMF isolate. It should be noted that in these particular experiments both glycoproteins and glycolipids would be labeled with the glucosamine and fucose and would be taken into account in determining the specific activity.

[^{125}I] Concanavalin A ([^{125}I] Con A) can be used to detect glycopeptides which contain mannosyl residues in their carbohydrate chains even after the glycopeptides have been separated via SDS-PAGE.[102] Figure 13 is an autoradiograph on which the UMF and WPM Con A receptors are displayed. There are approximately 20 Con A binding glycopeptides in the WPM but only ∿6 Con A binding glycopeptides in the UMF. These results agree with the above data which suggest an enrichment of glycoproteins in the WPM as compared to the UMF.

Role of Microtubules and Microfilaments in Determining Membrane Protein Topography

In order to investigate the possible roles of microtubules and microfilaments in the maintenance or determination of the UMF composition, cells were treated with agents which disrupt these cytoskeletal

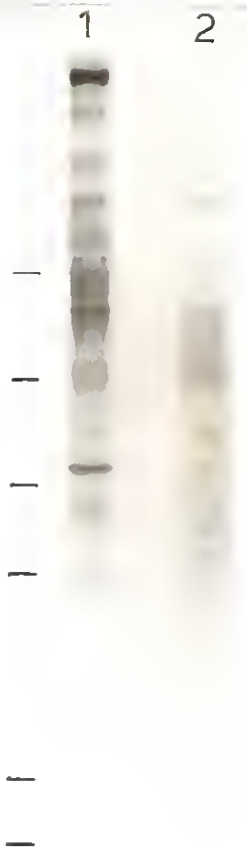

Figure 13. [^{125}I] Con A binding glycopeptides of UMF and WPM
Autoradiograph of [^{125}I] Con A binding glycopeptides of UMF and WPM. UMF and WPM were isolated from sptr 3T3 cells, solubilized in Laemmli sample buffer[97], and components separated on a 7.5-12.5% SDS-PAGE. Following staining, the gel was overlayed with [^{125}I] Con A according to a modification of the technique of Burridge.[102] The Con A binding glycopeptides were displayed by autoradiography. From left to right: markers corresponding to the molecular weight standards shown in Figure 4; Lane 1, WPM; lane 2, UMF.

elements. Following treatment with vinblastine sulfate (2.5×10^{-7}M, 2 hours, room temperature) to disrupt microtubules or cytochalasin B (8×10^{-6}M, 45 minutes, room temperature) to disrupt (or at least disorder) microfilaments, the UMF was isolated from the treated sptr 3T3 cells and compared, via SDS-PAGE, to UMF isolated from untreated cells. As can be seen in Figure 14, pretreatment of the sptr 3T3 cells with vinblastine sulfate or cytochalasin B before UMF isolation did not alter the coomasie blue staining SDS-PAGE profile.

Pretreatment of the cells with these agents was also found to have no effect on the yield of UMF from the cells. These data suggest that microtubules and microfilaments are not responsible for maintaining the distribution of membrane components, since disruption of these cytoskeletal elements does not alter the UMF composition.

Figure 14. Effects of vinblastine sulfate and cytochalasin B treatments on the UMF.

Coomasie blue staining profile of UMF peptides and glycopeptides separated on a 7.5-12.5% SDS-PAGE. From left to right: markers corresponding to the molecular weight standards shown in Figure 4; Lane 1, UMF isolated from sptr 3T3 cells treated for 45 minutes at room temperature with 9 x 10^{-6}M cytochalasin B; lane 2, UMF isolated from sptr 3T3 cells treated for 2 hours at room temperature with 2.5 x 10^{-7}M vinblastine sulfate; lane 3, UMF isolated from untreated sptr 3T3 cells.

DISCUSSION

In the work presented in this dissertation we have defined and developed the membrane isolation technique first introduced by Barland and Schroeder.[91] We have done this in the hope of developing a technique for isolating the upper surface membrane of cells attached to an artificial substratum in order to characterize the biochemical composition of the upper surface of the plasma membrane. Our long range goal is, of course, to correlate the unique composition of this upper compartment of the plasma membrane with the physiologic properties which have been associated with the dorsal surface of substratum attached cells.

Using the technique of scanning electron microscopy, we have been able to examine the sptr 3T3 cells used in these experiments at each step of the UMF isolation procedure. These cells have a heterogeneous morphology, with both fibroblastic and epithelioid cells being present in about equal numbers in cultures maintained in medium supplemented with 10% calf serum. Within a culture the individual cells are smooth surfaced with few microvilli. As we have demonstrated, the solutions used in the UMF isolation procedure cause very obvious changes in surface topography on these cells. The $ZnCl_2$/DMSO treatment of the sptr 3T3 cells produces blebbing of the cell surface, regardless of the individual cell's morphology. The "fixation" in $ZnCl_2$/DMSO also causes some stretching of the upper surface of the epithelioid cells, which is probably responsible for the appearance of holes in the upper surface of the cells at this step in the membrane isolation.

The blebs which appear during the $ZnCl_2$/DMSO incubation are absent from cells which have subsequently been incubated 40 minutes in ice-cold saturated FMA. Furthermore during the FMA incubation, the number of holes in the dorsal surface of the cells increases. Both the loss of blebs and the increase in the number of holes in the upper cell surface occur after FMA incubation whether the cells are agitated or not. Shaking of the FMA treated cells is, however, necessary to achieve a release of large sheets of membrane into the FMA solution. The loss of sheets of membrane from the substratum attached cells is much more common in the epithelioid, as opposed to the fibroblastic, cell population.

As mentioned, shaking of the cells during the FMA treatment releases a portion of the upper membrane from a proportion of the cells within the population. Data derived from scanning electron microscopy suggest that the UMF is removed from the area of the upper surface circumscribing the nucleus. Following UMF removal plasma membrane remains associated with the ventral surface of the cell "remains" as well as being retained directly over the nucleus and on the cell periphery.

Examination by phase microscopy shows that following agitation of the cells in FMA large sheets of membranous material are released into the FMA solution. Transmission electron micrographs of the purified UMF reveal a plasma membrane isolate virtually free of contamination by vesicles or adherent cytoplasmic material. The purity of this membrane isolate is also reflected by the low inclusion of DNA or RNA in the membrane pellet.

The plasma membrane which remains associated with the substratum after the UMF has been removed has been isolated and purified. We had hoped that the purification of this membrane fraction, designated as membranes associated with the substratum (MAS), would allow us to directly compare the composition of plasma membranes from two well-defined areas of substratum-attached cells. As we discovered, however, only the epithelioid cells within the sptr 3T3 cell population (which comprise approximately 50% of the cells in the population) release a portion of their upper membranes during the UMF isolation procedure. Fibroblastic cells retain their upper surface membranes throughout the membrane isolation procedure and therefore contribute both dorsal and ventral surfaces to the material isolated as MAS. Thus, although the UMF represents plasma membranes removed from a specific, well-defined area on the cell surface, the MAS contains membrane from all areas of the cell surface.

Although it is clear that the UMF is only isolated from the epithelioid cells within this cell population, it is not clear what property of these epithelioid cells makes them susceptible to the action of the tanning reagents. At least two explanations for the preferential effect on epithelioid cells exist: 1) the cell morphology affects the $ZnCl_2$/DMSO-FMA "fixation" process or 2) the plasma membranes are "fixed" equally in all cells regardless of morphology but the effects of fixation are manifest more dramatically in the more spread out cells. The first of these possibilities implies that differences exist between the epithelioid and fibroblastic upper membrane surfaces before any treatment and these differences mediate the effects of the $ZnCl_2$/OMSO-FMA treatments. This possibility is difficult

to test; however, it must be noted that blebbing occurs in both types of cells following $ZnCl_2$/DMSO treatment, although only the epithelioid cells have extensive tearing of the upper surface. The second possibility suggests that the widespread substratum attachments of the epithelioid cells cause shrinkage of the upper membrane surfaces thereby producing holes in that surface. According to this hypothesis the more cylindrical shape of the fibroblast cells allows shrinkage of the plasma membrane to occur with less stress and subsequently without tearing holes in the upper membrane.

As indicated in the Results section, the yield of membrane protein in the UMF is approximately 4% of the total cellular protein. One might be tempted to use this value to extrapolate the percent cellular protein in the total plasma membrane. Assuming that the UMF accounts for about half of the plasma membrane from those cells from which it is removed, the whole plasma membrane from these cells would be about 8% of the total cellular protein. Since roughly 50% of the cells release an UMF, a value of 16% membrane protein would be necessary if one were to account for total plasma membrane from all cells of the population. It is our opinion, however, that such a calculation will yield spurious numbers. As we have demonstrated, the UMF is a very protein rich/lipid poor compartment of the cell. Thus, since the UMF represents an area of cell surface enriched in protein, any attempt to calculate the total plasma membrane protein based on UMF protein yields is undoubtedly going to yield incorrect and inflated values.

Our data clearly indicate compositional differences among the UMF, MAS, and WPM membrane isolates. Coomasie blue staining of SDS-PAGE profiles of the membrane peptides and glycopeptides shows that the

compositional differences are most striking in the high molecular weight (MW > ~120,000) region of the gel. Many of the high molecular weight species are greatly reduced in the UMF profile (as compared to the WPM profile) but present in exaggerated amounts in the MAS. This evidence strongly suggests that these high molecular weight membrane constituents are localized to the periphery and underside of the cell surface.

Using [^{3}H] glucosamine and [^{3}H] fucose to metabolically label glycoproteins, as well as [^{125}I] Con A, $NaIO_2$/NaB[^{3}H]$_4$ and galactose oxidase/NaB[^{3}H]$_4$ to directly label mannosyl, sialic acid, and galatosyl moieties respectively, we have shown that the UMF is a region of the plasma membrane which is glycoprotein-poor as compared to other areas on the cell surface. In addition, we have shown, using the lactoperoxidase-catalyzed iodination of the cell surface, that the UMF contains relatively few iodinateable components. It is particularly noteworthy that the UMF contains substantially less LETS[28,29] protein per mg protein than does either the WPM or MAS. This finding is in agreement with other studies[30-33] demonstrating that LETS is localized to the periphery and underside of cells grown in vitro.

By isolating UMF from the cells maintained in medium supplemented with [^{3}H] leucine, we have demonstrated that the UMF is clearly a product of cellular metabolism. In addition our results indicate that the UMF is not an isolate composed of adherent serum components, collagen, or sulfated glycosaminoglycans.

Our data suggest that the unique composition of the UMF does not result from extraction of membranous components by $ZnCl_2$/OMSO or FMA. Although blebs which appear during the $ZnCl_2$/OMSO steps are largely

gone after a 40 minute incubation in FMA at 0°C, none of our experiments have suggested that the blebs are released from the membrane into the FMA solution. Furthermore our results suggest that the high molecular weight peptides and glycopeptides missing from the SDS-PAGE profile of the UMF are not extracted into the FMA solution.

With regard to the relatively high protein/lipid ratio observed in the UMF, we have demonstrated that the $ZnCl_2$/DMSO-FMA solutions do not extract lipid from isolated membranes and that low density lipid vesicles are not released into the $ZnCl_2$/DMSO or FMA solutions. We have not directly ruled out the possibility that lipid vesicles containing other material (e.g. cytoplasm) and having a density greater than that of 55% (w/w) sucrose could be separated from the cell surface and then, despite their very high density, not pellet through the washes or 50% (w/v) sucrose gradient of the UMF isolation procedure. We consider the possibility unlikely, however, because a portion of these high density vesicles would be expected to pellet with the UMF. Our transmission electron micrographs show no evidence of any contamination of the UMF with vesicular or cytoplasmic material.

A number of possibilities exist to explain the distinct compositional differences which have been observed between the UMF and WPM. Perhaps the most interesting possibility (and the one which has been the impetus behind this dissertation) is that the area of the membrane isolated by the Barland and Schroeder[91] technique is physiologically distinct from the remainder of the surface membrane, and that the peptide, glycopeptide, and lipid composition of the UMF reflect an area of plasma membrane "differentiated" to perform special functions. A second possible explanation for the compositional differences between UMF and WPM is that a specific membrane composition is a

prerequisite for the efficient release of membrane by the $ZnCl_2$/DMSO-FMA treatment. According to this hypothesis, the morphology of the cell might influence, if not determine, the biochemical composition of the upper membrane. Thus, epithelioid cells might have a different upper membrane composition than the fibroblastic cells, and this difference itself might make the upper membrane of this epithelioid cells more susceptible to removal by the shearing action of the FMA solution. The third possible explanation for the observed composition of the UMF is that treatment of the sptr 3T3 cells with $ZnCl_2$/DMSO-FMA rearranges or repositions membrane proteins, glycoproteins, or lipids within the lateral plane of the membrane. If this third hypothesis was correct, the compositional differences observed between the UMF and WPM might be artifacts of the $ZnCl_2$/DMSO-FMA fixation. This latter hypothesis must be seriously considered in light of recent evidence[18] suggesting that crosslinking of surface constituents through disulfide bonds may act to stabilize membrane peptides and glycopeptides, since the FMA is known to bind to sulphydryl bonds.

It is obvious that the first two hypotheses are much more exciting in light of their impact on our understanding of the nature of plasma membrane structure. These hypotheses and the data generated in this dissertation to support these hypotheses tentatively suggest that membrane constituents are compartmentalized and that the composition of the dorsal surface of the plasma membrane differs from the composition of other areas of the plasma membrane.

The results of our experiments give us a number of clues as to what this upper surface membrane compartment is like, at least in the sptr 3T3 cells of epithelioid morphology. Our data strongly suggest

that this area of the cell surface is packed very densely with membrane proteins, as compared to the other cell surface regions. High molecular weight peptides are excluded from this upper membrane compartment, as are the majority of glycopeptides of all molecular weights. Interestingly, many of the proteins which are found in the upper membrane fraction are not accessible to surface labeling techniques, including lactoperoxidase catalyzed [^{125}I] labeling. This suggests that the tyrosine moieties of the UMF proteins are not available to the lactoperoxidase, a finding which is surprising.

At the present time, we have no clear indication of the mechanisms responsible for the maintenance of a distinct composition on the dorsal surface of the plasma membrane. One very exciting possibility is that the membrane proteins are packed into this upper surface region by processes similar (or identical) to those responsible for the movement of particles attached to cells at the periphery. Albrecht-Buehler[34] has demonstrated, and we have observed in our laboratory, that gold particles attached to the dorsal surface of the cells move from the cell periphery toward the nucleus (centripetal flow). Centripetal flow of other types of particles attached to the cell surface has been demonstrated in other laboratories.[58,59,72,79-82] This centripetal flow results in the clustering of particles circumferentially around (but not over) the nucleus in a ring which bears a striking resemblance in its location on the upper surface to the most central area of UMF removal. If centripetal flow of membrane is responsible for the composition of the UMF, our results imply that the flow is specific in terms of composition as well as direction. High molecular weight peptides and most glycopeptides would have to be excluded from this movement.

The work of DiPasquale and Bell[83] has demonstrated (see Introduction) variations in the capacity of differing cell surface regions to form attachments with other cells. Specifically, they have shown that the upper surface will not support spreading or attachment of other cells. One could postulate that the paucity of carbohydrate-bearing proteins on the upper surface, as well as the relative absence of exposed, iodinateable surface protein moieities could be related to the altered attachment properties of this area of the surface membrane.

Our data do suggest that a plasma membrane compartment (the UMF) does exist and that this area has a unique composition and is positioned in a well-defined area on the substratum attached cells we have used for these experiments. These experimental findings indicate that current plasma membrane models may have to be expanded to include discrete, but large, compositionally distinct regions within the lateral plane of the membrane.

In order to establish more completely the nature of plasma membrane compartmentalization, specific elements of the plasma membrane must be identified which are unequivocally localized to one region of the cell surface. A localization of this sort would immediately verify the existence of membrane compartmentalization, if not totally confirm our interpretation of the UMF isolation procedure. We hope to investigate such a possibility in ongoing experiments using antibodies directed against lectin receptors. From our [^{125}I] Con A binding data, we have established that a number of Con A receptors are localized to the areas of the surface not isolated as UMF. It is our hope to prepare antibodies against one of these specific receptors.

Using fluorescent conjugates of such antibodies, we should be able to identify this receptor on the surface of intact, untreated cells. Hopefully such experiments would unequivocally demonstrate the compartmentalization of specific membrane constituents in substratum attached cultured cells.

In summary, we believe that the UMF isolation technique, originally introduced by Barland and Schroeder[91] allows the isolation of a biochemically and topographically distinct area of the plasma membrane. Of great importance to understanding ours and others work is the realization that the membrane fraction isolated by this technique is not representative of the structure of the plasma membrane from other areas of the cell. It is our opinion that, as a result of its selectivity with regard to cell morphology and area of the plasma membrane isolated, the Barland and Schroeder isolation procedure probably has only limited application to the study of generalized membrane structure. In light of these limitations, we would suggest that some of the data which has been collected by other laboratories[116,117] using the isolation technique should be reevaluated. However, we do believe that this technique provides an excellent tool for the isolation of a specific region on the cell surface. Thus, the UMF isolation technique may allow the study of the compartmentalization of cellular components and the effects of differing environments (substratum or medium) on defining and maintaining these compartments.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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